

INFLUENCE OF BODY CONDITION SCORE OF DAIRY COWS AT THE END OF PREGNANCY ON PERIPHERAL TISSUE INSULIN RESPONSE AND METABOLIC PROPERTIES OF ADIPOSE TISSUE

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**Influence of body condition score
of dairy cows at the end of pregnancy
on peripheral tissue insulin response
and metabolic properties of adipose tissue**

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Dissertation submitted in fulfillment of the requirements for the degree of Doctor of

Philosophy (PhD) in Veterinary Sciences

2016

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This research was funded by the Special Research Fund of Ghent University, grant number 01D28410.

Dit onderzoek werd gesubsidieerd door het Bijzonder Onderzoeksfonds van de Universiteit Gent, beursnummer 01D28410.

Layout by Rien De Koster

Printed by University Press, Zelzate

Cover design by Maarten Hermans

Printing of this thesis was financially supported by



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**Remember the Past and Plan for the Future but Live for Today
because Yesterday is Gone and Tomorrow may Never Come**

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List of Abbreviations

AA	Amino acids
ACC	Acetyl CoA carboxylase
Acetyl-CoA	Acetyl coenzyme A
ADF	Acid detergent fiber
ADL	Acid detergent lignin
ADP	Adenosine diphosphate
AIRg	Acute insulin response to glucose derived from the minimal model
AMPK	Adenosine monophosphate-activated protein kinase
ATGL	Adipose triglyceride lipase
ATM	Adipose tissue macrophages
ATP	Adenosine triphosphate
AUC	Area under the curve
BCS	Body condition score
BFT	Backfat thickness
BHB	β -hydroxybutyrate
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCC	Concordance correlation coefficients
CD14	Cluster of differentiation 14
CGI-58	Comparative gene identification 58
CO ₂	Carbon dioxide
CP	Crude protein
CPT-1	Carnitine palmitoyltransferase 1
CR	Clearance rate
CV	Coefficient of variation
DI	Disposition index derived from the minimal model
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake

EC50	Effective concentration to achieve the halfmaximal effect
ED50	Effective dose to achieve the halfmaximal effect
FAS	Fatty acid synthetase
G0	Postinjection glucose concentration derived from the MINMOD
Gb	Basal glucose concentration derived from the MINMOD
GH	Growth hormone
GIR	Glucose infusion rate
GLUT	Glucose transporter
GPAT	Glycerol-3-phosphate acyl transferase
GTT	Glucose tolerance test
HEC test	Hyperinsulinemic euglycemic clamp test
HOMA	Homeostasis model assessment
HOMA-IR	Homeostasis model assessment of insulin resistance
HSL	Hormone sensitive lipase
IGF-1	Insulin like growth factor-1
IL6	Interleukin 6
IRS-1	Insulin receptor substrate-1
ISI	Insulin sensitivity index
ISO	Isoproterenol hydrochloride
IVGTT	Intravenous glucose tolerance test
IVITT	Intravenous insulin tolerance test
LDA	Left displacement of the abomasum
LPL	Lipoprotein lipase
MCP-1	Monocyte chemoattractant protein-1
MGL	Monoacylglycerol lipase
mRNA	Messenger ribonucleic acid
NADH	Reduced nicotinamide adenine dinucleotide
NDF	Neutral detergent fiber
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NE _l	Net energy for lactation
PDE-3B	Phosphodiesterase 3B

PKA	Protein kinase A
PPAR α	Peroxisome proliferator-activated receptor α
QUICKI	Quantitative insulin sensitivity check index
R _{max}	Maximal biological effect
RQUICKI	Revised quantitative insulin sensitivity check index
RQUICKI _{BHB}	Revised quantitative insulin sensitivity check index including BHB
RQUICKI _{glycerol}	Revised quantitative insulin sensitivity check index including glycerol
RT-qPCR	Quantitative reverse transcriptase real-time polymerase chain reaction
Sg	Glucose effectiveness derived from the minimal model
SGLT	Sodium dependent glucose transporter
Si	Insulin sensitivity index derived from the minimal model
SS	Steady state
SSGIR	Steady state glucose infusion rate
SSIC	Steady state insulin concentration
SSNEFA	steady state NEFA concentration
TAG	Triacylglycerol
TG	Triglycerides
TNF	Tumor necrosis factor
VFA	Volatile fatty acids
VLDL	Very low density lipoproteins

CHAPTER 1

Pregnancy and lactation in the dairy cow, a quest for glucose

Adapted from: De Koster J. and Opsomer G. 2013. Insulin resistance in dairy cows. Veterinary Clinics of North America: Food Animal Practice. 29:299-322.

1. THE CIRCLE OF LIFE

Glucose, a molecule consisting of six carbon atoms, twelve hydrogen atoms and six oxygen atoms ($C_6H_{12}O_6$), plays an essential role in the metabolism of most living organisms on earth (Sjaastad et al., 2010). It is produced by plants during photosynthesis starting from water and carbon dioxide under the influence of energy by sunlight (Singhal et al., 2012). This glucose is stored under the form of polymers like starch and other complex carbohydrates (Bonner and Varner, 2012). Animals rely, for a large part, on the intake of carbohydrates by eating plants or animals for the provision of glucose as substrate for energy production. Alternatively, they can synthesize glucose via the gluconeogenic pathway in the liver and the kidneys.

Energy for cellular metabolism of animals is generated by the dissociation of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and phosphate, a reaction which generates approximately 12 kcal/mol (Cooper, 2000). Adenosine triphosphate is generated during oxidative phosphorylation, the Krebs cycle and glycolysis. Oxidative phosphorylation takes place in the mitochondria and consists of a series of redox reactions starting from NADH (reduced nicotinamide adenine dinucleotide). During oxidative phosphorylation, carbon dioxide is produced which is excreted and can be used by plants to produce glucose during the photosynthesis. NADH is generated during the Krebs cycle and the glycolysis. The Krebs cycle takes place in the mitochondria and uses acetyl-CoA as starting molecule which can be produced by the breakdown of glucose during glycolysis, the breakdown of fatty acids via the β -oxidation pathway and the breakdown of amino acids. The glycolytic pathway takes place in the cytoplasm and breaks down glucose into two molecules of pyruvate which generates energy in the form of ATP and NADH. The sequential breakdown of energetic compounds starting from one molecule of glucose in the glycolytic pathway, the Krebs cycle and oxidative phosphorylation generates 36 to 38 molecules of ATP (Figure 1) (Cooper, 2000; Sjaastad et al., 2010). Despite the fact that lipids and proteins can be used as energy source, certain vitally important cell types (erythrocytes, brain- and kidney cells) rely on glucose as the only energy substrate (Aschenbach et al., 2010). Therefore, maintenance of blood glucose levels within normal physiological ranges is of utmost importance in all living mammals (Vidal-Puig and O'Rahilly, 2001).

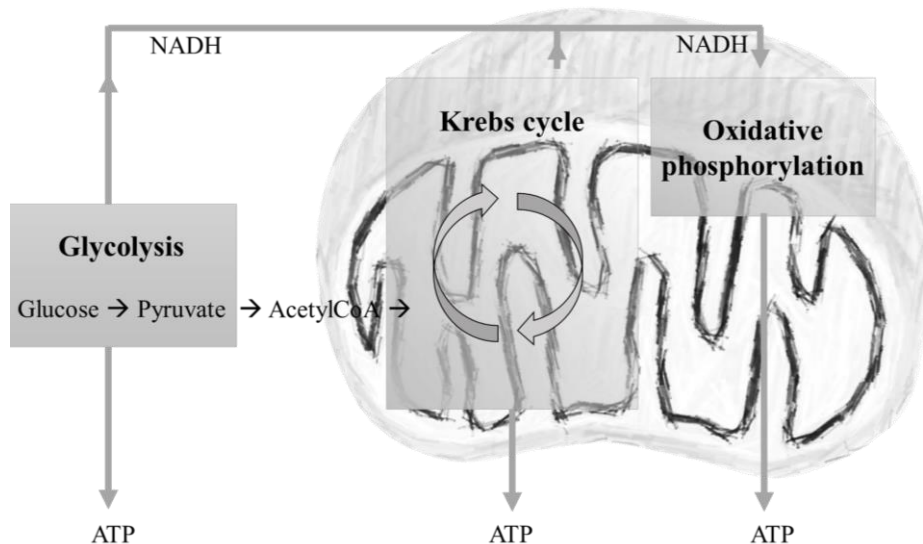


Figure 1: Overview of the sequential breakdown of glucose for the generation of energy for cellular metabolism in the glycolytic pathway, the Krebs cycle and the oxidative phosphorylation.

2. COWS ARE RUMINANTS AND POSSESS A VERY SPECIFIC GLUCOSE METABOLISM

Dairy cows are ruminants and like other herbivores, they are able to digest plant fiber. The digestion of this fiber takes place in the well-developed forestomachs of ruminants: the rumen, the reticulum and the omasum. Within the reticulum and the rumen, the feed particles are fermented by the ruminal flora which consists mainly of bacteria and protozoa (Sjaastad et al., 2010). The bacteria and protozoa attach to the ingested feed particles and start to degrade and/or utilize the available feed components: structural and non-structural carbohydrates, protein, non-protein nitrogen and fats (Lean et al., 2014). The so called ruminal fermentation of the different components by the bacteria and protozoa yields methane, volatile fatty acids (mainly propionate, acetate and butyrate) and microbial protein. When the ingested feed particles are small enough due to the degradation by the ruminal flora and the process of rumination, they continue their way through the digestive tract. In the omasum, water is absorbed and particle size is reduced before the feed components enter the abomasum, where digestive enzymes are secreted to continue the digestion of the feed components (Sjaastad et al., 2010). Due to the vigorous process of fermentation in the forestomachs, very little glucose is available for direct absorption in the gastrointestinal tract (Aschenbach et al., 2010). However, the amount of glucose absorbed in the small intestine is strongly dependent on the total amount of starch in

the diet, the grain source of the ingested starch and the processing of the grain (Moharrery et al., 2014). Nevertheless, most of the circulating glucose in ruminants originates from hepatic and renal gluconeogenesis, where the liver contributes to more than 80% of the total glucose supply in sheep (Aschenbach et al., 2010). As a consequence, the glucose metabolism of ruminants is characterized by low peripheral glucose concentrations (Annison and White, 1961; Kaske et al., 2001; Hayirli, 2006) and a low insulin response of the peripheral tissues (Brockman and Laarveld, 1986; Bell and Bauman, 1997; Kaske et al., 2001; Sasaki, 2002). The glucose metabolism of ruminants is regulated by the supply and removal of glucose and glucogenic precursors in the blood and is tightly controlled by different hormones. Within the ruminants, dairy cows occupy a special position considering the glucose metabolism. The massive glucose drain towards the udder and the unique transition from pregnancy to lactation makes glucose metabolism of dairy cows an example of how intensive genetic selection can drive metabolism into extremes. A schematic overview of the glucose metabolism in dairy cows is given in Figure 2.

3. GLUCOSE SUPPLY IN DAIRY COWS

3.1. Propionate

As stated above, most of the circulating glucose in ruminants originates from hepatic and renal gluconeogenesis. Different endo- and exogenous substrates are used for this gluconeogenesis. In the forestomachs, short-chain fatty acids are formed by microbial fermentation of carbohydrates in the feed. Among these short-chain fatty acids; propionate, isobutyrate and valerate are the main contributors for gluconeogenesis (Aschenbach et al., 2010). The relative contribution of glucogenic precursors changes during the different stages of lactation depending on feed intake, feed composition, tissue mobilization and energy balance. Quantitatively, propionate (60-74%) is the most important glucogenic precursor followed by lactate (16-26%), alanine (3-5%), valerate and isobutyrate (5-6%), glycerol (0.5-3%) and other amino acids (8-11%) (Drackley et al., 2001; Aschenbach et al., 2010).

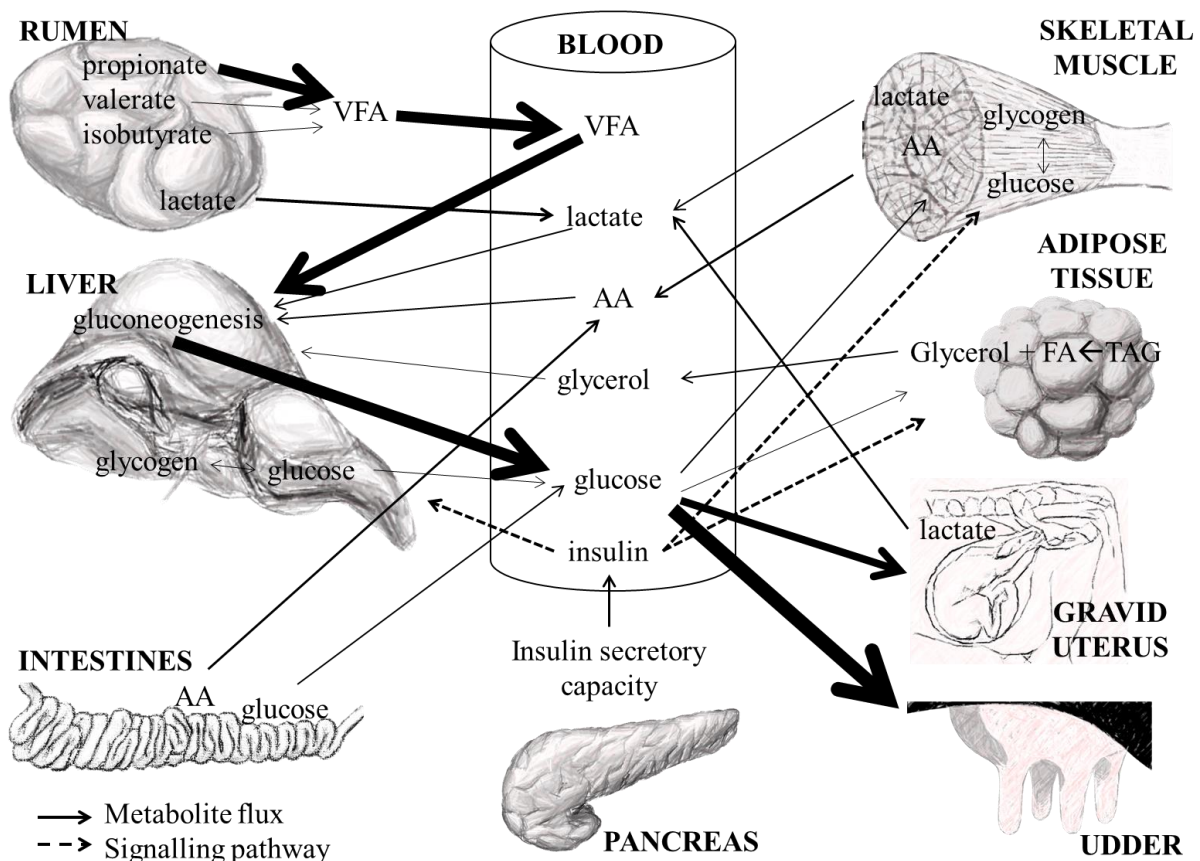


Figure 2: Schematic overview of the glucose metabolism in dairy cows. Thickness of arrows indicates the importance of the metabolite or tissue in production or utilization. Volatile fatty acids (VFA) produced in the rumen are the most important precursors for hepatic gluconeogenesis. In addition, lactate (originating from the rumen, skeletal muscles and gravid uterus), glycerol (from lipolysis of triglycerides (TAG) in the adipose tissue) and amino acids (AA from the intestines and skeletal muscles), all contribute to the total hepatic gluconeogenesis. Glucose absorbed from the intestines and glucose released from the liver (mostly gluconeogenesis, partly glycogenolysis) are the most important contributors to the blood glucose level. The lactating udder and the gravid uterus are quantitatively the most important glucose consumers. In the dry period and during early lactation, skeletal muscles and adipose tissues take up minimal amounts of glucose. The pancreas releases insulin into the bloodstream which suppresses hepatic gluconeogenesis, glycogenolysis in liver and skeletal muscles and adipose tissue lipolysis, whereas insulin stimulates glucose uptake in skeletal muscles and adipose tissues.

3.2. Lactate

Lactate can have an endo- or exogenous origin. Rations based on high amounts of concentrates, provoke a shift from a cellulolytic to an amylolytic flora in the rumen (Slyter, 1976). This amylolytic flora is responsible for the production of lactate in the rumen, which can be utilized by the liver for glucose production (Nocek, 1997). However, an overproduction of lactate may become detrimental for the health of the cow due to its low pKa and concomitant higher risk to suffer from (sub)-clinical ruminal acidosis. Another source of lactate is the anaerobic oxidation of glucose in the skeletal muscles and other peripheral tissues. During early lactation, expression of lactate dehydrogenase, the enzyme responsible for converting pyruvate into

lactate, is up regulated whereas the expression of the main enzymes of the Krebs cycle are down regulated in skeletal muscle. The latter indicates that the catabolism of glucose in skeletal muscle is diverted towards lactate production indirectly supporting gluconeogenesis (Kuhla et al., 2011). At the end of pregnancy, the uterus and placenta are important sources of lactate (Bell, 1995). Therefore, the relative contribution of lactate in hepatic gluconeogenesis is maximal at the end of pregnancy and during early lactation (Drackley et al., 2001; Reynolds et al., 2003).

3.3. Glycerol

When adipose stores are mobilized, non-esterified fatty acids (NEFA) and glycerol are released into the bloodstream. The fate of the NEFA is well described in other reviews (Drackley, 1999; Adewuyi et al., 2005). The released glycerol can be used in the gluconeogenic pathway. Its contribution to gluconeogenesis and hence to the overall glucose production is dependent on the size of the fat depots and the amount of fat mobilization and is thus directly related to the negative energy balance (Drackley et al., 2001).

3.4. Amino acids

Circulating amino acids provide another source to be used in gluconeogenic pathways, especially during periods of high glucose requirements. The extra amino acids originate from intestinal absorption (higher feed intake), decreased protein synthesis in skin and skeletal muscles and increased protein breakdown in skeletal muscles (Bell et al., 2000; Drackley et al., 2001). The most important amino acids to contribute in glucose provision are alanine and glutamine (Drackley et al., 2001).

3.5. Glycogen

Glycogen stores in the liver and skeletal muscles provide a store of glucose in the body of the dairy cow. In periods of low glucose availability or high glucose requirements (end of pregnancy and early lactation), these stores can be mobilized. Only the glycogen stores in the liver can directly support the blood glucose level, since the liver is able to convert glucose 6-phosphate into glucose. Skeletal muscles lack the enzyme, glucose 6 phosphatase, necessary for this conversion and therefore glycogen stores from skeletal muscle do not directly contribute to the blood glucose level (Cox and Nelson, 2004). Due to the rather limited size of the

glycogen stores in the liver, this glucose reserve is regarded a minor contributor to the overall blood glucose regulation at the end of pregnancy and at the beginning of lactation (Veenhuizen et al., 1991; Herdt, 2000; Karcagi et al., 2008). Glycogenolysis and glycolysis in skeletal muscle are up regulated at the initiation of lactation. In this period, the oxidation of glucose in skeletal muscle is shifted in the direction of lactate. Since lactate can be converted to glucose by the liver, this shift in glucose metabolism within the skeletal muscle means that the muscular glycogen stores contribute indirectly to the blood glucose levels (Kuhla et al., 2011).

4. GLUCOSE REMOVAL IN DAIRY COWS

4.1. Glucose uptake: a facilitated process

Glucose cannot pass the plasma membrane surrounding the cells (Shepherd and Kahn, 1999). The glucose uptake takes place by 2 different processes: facilitated diffusion or cotransport. The cotransport is mediated by sodium dependent glucose transporters (SGLT) and is driven by a difference in sodium concentration between the intra- and extracellular fluid. The sodium dependent glucose transporters are located at the epithelial cells of the small intestine and the tubulus cells of the kidney (Zhao and Keating, 2007). Most cells however take up glucose by facilitated diffusion via glucose transporter (GLUT) molecules. The uptake of glucose through these GLUT is basically driven by the difference in glucose concentration between the extra- and intracellular fluid (Sasaki, 2002; Zhao and Keating, 2007). There are 13 different isoforms of the GLUT, all of them having a specific tissue distribution, expression profile, and specific properties regarding sensitivity to hormones. The GLUT1 molecule is expressed in all tissues throughout the body and is responsible for the basal glucose uptake (Zhao and Keating, 2007). Of all the GLUT molecules, GLUT4 is the only one that is responsible for the insulin stimulated glucose uptake in skeletal muscle, heart and adipose tissue (Zhao and Keating, 2007). Quantitatively, the most important glucose consuming tissues are the skeletal muscles, the udder and the gravid uterus.

4.2. Glucose uptake in skeletal muscle and adipose tissue

In skeletal muscle and adipose tissue, glucose is transported into the cells by GLUT1 and GLUT4 molecules. GLUT1 accounts for the basal glucose supply while GLUT4 mediates the insulin stimulated glucose uptake (Duhlmeier et al., 2005; Zhao and Keating, 2007). Upon

insulin stimulation, intracellularly stored GLUT4 are translocated to and fused with the plasma membrane. The increased number of GLUT4 on the cell membrane are responsible for the insulin induced blood glucose reduction (Shepherd and Kahn, 1999). The previously mentioned lower insulin response of skeletal muscle and adipose tissue in ruminants when compared to monogastric animals, is partly due to the lower number and the lower insulin induced translocation of GLUT4 in these tissues in ruminants (Sasaki, 2002; Duhlmeier et al., 2005). Another typical feature in ruminants is that the adipose tissue prefers to use acetate, a VFA produced in the forestomachs, as substrate for lipogenesis. Monogastrics use glucose for this purpose (Hanson and Ballard, 1967; Brockman and Laarveld, 1986). Therefore, the adipose tissues in ruminants account for only a small part of the total insulin induced glucose disposal.

In order to preserve sufficient glucose for fetal growth and development, homeorhetic changes of the glucose metabolism take place throughout the body during pregnancy. At the level of the skeletal muscle and adipose tissue, glucose consumption is reduced. According to Komatsu et al., GLUT4 mRNA expression was not altered in adipose tissue or skeletal muscle during lactation or dry period in dairy cows (Komatsu et al., 2005). A recent study using western blot analysis of skeletal muscle however revealed that GLUT4 content was reduced by 40% by the 4th week in lactation in comparison to the dry period (Kuhla et al., 2011), suggesting a post-transcriptional regulation of GLUT4 mRNA in the skeletal muscle in order to reduce muscular glucose uptake in early lactation.

In adipose tissue, expression and protein content of GLUT1 and GLUT4 are minimal during peak lactation and increase at the end of lactation to remain elevated during the dry period (Komatsu et al., 2005; Sadri et al., 2010; Ji et al., 2012). This expression profile gives rise to a decreased basal and insulin stimulated glucose uptake by the adipose tissue in early lactation thereby sparing glucose for milk production.

4.3. Glucose uptake by the uterus, placenta and fetus

The greatest increase in fetal mass and fetal nutrient requirements occurs during the last 2 months of pregnancy (Bauman and Currie, 1980). Glucose and amino acids are the most important energy sources for the development of the fetus, the uterus and the placenta (Bell et

al., 1995). The GLUT molecules responsible for the glucose uptake in the ovine placenta are of the GLUT1 and GLUT3 isoform type (Bell and Bauman, 1997). Only during the last trimester of pregnancy, uterine, fetal and placental glucose requirements substantially increase the total glucose requirement of the dam (Bauman and Currie, 1980). In sheep, this is associated with an increased number of GLUT3 in the placenta (Bell and Bauman, 1997).

In cattle, placental expression of GLUT1, GLUT3, GLUT4 and GLUT5 has been demonstrated (Bertolini et al., 2004; Hirayama et al., 2011; Lucy et al., 2012). The expression of GLUT4 mRNA in placental tissue during early pregnancy is a new finding in dairy cows and had been demonstrated before in humans. Although no direct evidence exists that insulin stimulates the glucose uptake by placenta in humans or cattle (Hay Jr, 2006; Lucy et al., 2012).

4.4. Glucose uptake by the mammary gland

The glucose consumption of the mammary gland in dairy cows is responsible for 50 to 85% of the whole body glucose consumption and amplifies the glucose demand by 2.5 at the third week of lactation in comparison to the demands during the end of the dry period (Drackley et al., 2001; Zhao and Keating, 2007; Lemosquet et al., 2009). Seventy two grams of glucose are needed to produce 1 kg of milk (Kronfeld, 1982). This glucose is transported into the epithelial cells of the alveoli and converted into lactose, creating an osmotic pressure which ultimately determines the amount of milk produced. The uptake of glucose into the bovine mammary gland is regulated by GLUT1, GLUT8, GLUT12, SGLT1 and SGLT2 (sodium dependent glucose transporter) (Zhao and Keating, 2007), GLUT1 being the most important. Its expression and protein content is almost undetectable during the dry period and increases several folds at the initiation of lactation (Komatsu et al., 2005; Zhao and Keating, 2007). The insulin independency regarding the mammary glucose uptake is further demonstrated by the absence of GLUT4 in the mammary gland (Komatsu et al., 2005).

4.5. Glucose uptake by the liver

In nonruminants, the liver is an appreciable glucose consumer (Hocquette et al., 1996). Due to the sophisticated carbohydrate metabolism, the main function of the ruminants' liver is glucose production with production rates increasing up to 3600 g per day and more during peak

lactation (Reynolds et al., 2003). The GLUT isoforms responsible for the transport of glucose out of (and into) the hepatocytes are of the GLUT2 and GLUT5 type (Hocquette et al., 1996).

5. QUANTITATIVE ASPECTS OF GLUCOSE METABOLISM IN DAIRY COWS

The total glucose requirement per day is the result of the combination of:

- Basal glucose requirement (1.57 mol/day);
- Glucose requirement of the gravid uterus (0.10 mol/kg fetus/day);
- Glucose requirement of the mammary gland (0.4 mol/kg milk).

The basal glucose requirement refers to the glucose required for the maintenance of basal functions of tissues in the body (brain, kidneys, intestines, skeletal muscle, heart, adipose tissue, intestines, liver, ...) and is minimal in early lactation (predicted average is 1.57 mol/day) (Danfaer, 1994; Overton, 1998). Data from Rose et al. indicate that basal glucose availability increases in mid lactating dairy cows (calculated at 2.6 mol/day at 205 DIM), probably because of the accretion of energy in adipose tissue (Rose et al., 1997).

The glucose requirement of the gravid uterus may be predicted as described by Overton (Overton, 1998), using quantitative data of glucose uptake by the gravid uterus in beef cows (Ferrell, 1991). Average glucose uptake by the gravid uterus (total of uterus, placenta and fetus) was 0.10 mol/kg fetus/day (Ferrell, 1991). By using this predicted glucose uptake and the predicted weight of the fetus in dairy cows during the last 90 days of pregnancy as described by Bell et al. (1995), it is possible to calculate a rough estimate of the glucose uptake by the gravid uterus in dairy cows.

The glucose requirement for milk synthesis is much better described in literature. *In vivo* estimates of glucose requirements for milk synthesis give values of 72 g glucose per kg milk produced (Kronfeld, 1982). This agrees well with the theoretically predicted value of 68.4 g per kg milk using a conversion efficiency of glucose into lactose from 73-74% (Danfaer, 1994). It seems that the efficiency of glucose conversion into lactose does not change with increasing milk production up to 35 kg milk per day (Danfaer, 1994).

The predicted total glucose requirement (in grams) per day during the last 30 days before parturition and the first 60 DIM is depicted in Figure 3. Assumptions made to calculate this

graph were: basal glucose requirements are the same in the dry period and during the first 60 DIM and, efficiency of milk production is the same in early lactation and peak lactation.

The glucose requirements increase sharply after parturition as milk yield increases steeply at that time, and are doubled by 7 days after parturition. For a cow producing 42 kg milk (Figure 3), glucose requirements are over 3 kg per day and 90% of this glucose is preserved for lactose production.

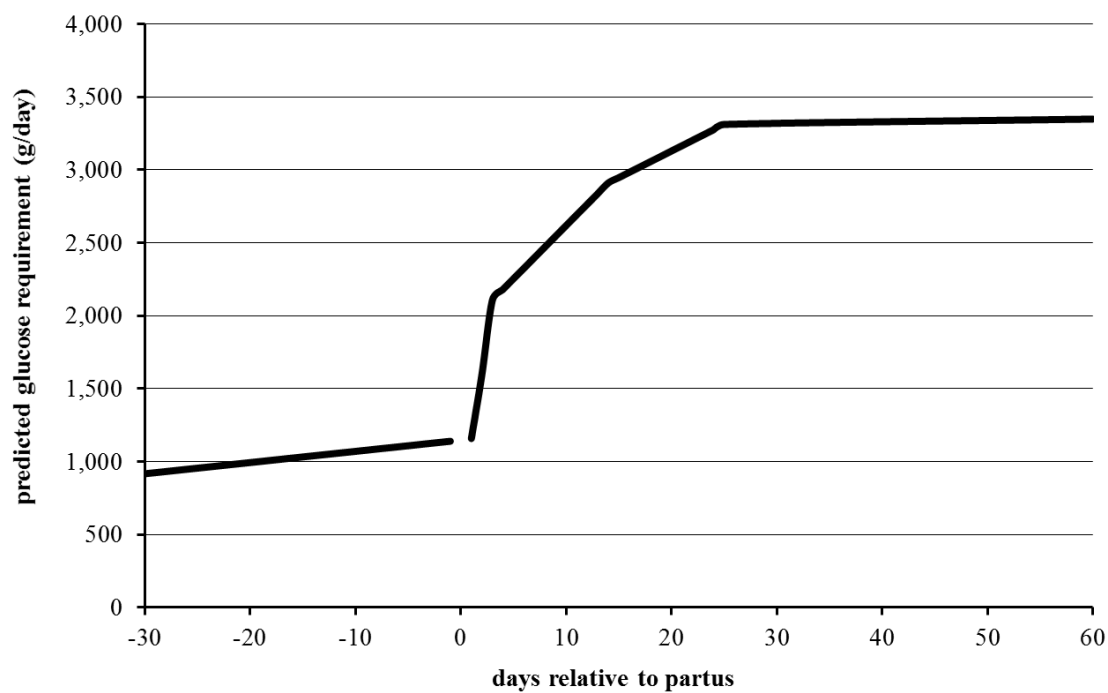


Figure 3: Predicted total glucose requirement in grams per day during the last 30 days before parturition and the first 60 DIM (42 kg milk). No prediction was made for the day of parturition because of the lack of data considering glucose requirement on that specific day. Glucose requirement calculated as described by Overton (1998).

This total glucose requirement can be divided in glucose uptake by the insulin sensitive tissues (skeletal muscle, heart and adipose tissue) and insulin insensitive tissues (mammary gland, uterus, brain, kidney,...). Only 8% of the total peripheral glucose uptake in midlactating dairy cows producing 26 kg milk is mediated by insulin (Rose et al., 1997). In humans, 25% of total glucose uptake is insulin dependent (DeFronzo, 2004). The large difference between humans and dairy cows is due to the insulin independent glucose uptake by the mammary gland of the latter (Rose et al., 1997).

6. PREGNANCY AND LACTATION, A QUEST FOR GLUCOSE

Pregnancy and lactation are two physiological states increasing the demands for glucose and very important for the survival of the species. In the dairy industry, these physiological states are elaborated for economic perspectives of the farmers. Due to intensive genetic selection, milk production has increased tremendously over recent years and exceeds the nutrient requirements of the neonate considerably. A suckling dairy calf will consume 8 to 12 L milk per day while peak milk production of dairy cows achieves easily 40 to 50 L of milk per day. For the periparturient dairy cow, it is impossible to meet its glucose needs due to the rapidly increasing milk yield, the periparturient drop in dry matter intake and the slow postpartum increase in dry matter intake. Inevitably, they go into a period of negative energy and glucose balance. To support the partitioning of glucose towards the pregnant uterus and the lactating mammary gland, substantial metabolic adaptations have to occur at the level of carbohydrate, protein and lipid metabolism to support the changing physiological environment at the end of pregnancy and the beginning of lactation.

The cow shifts her metabolism towards an increased glucose production, decreased glucose use by non-mammary tissues and increased use of energy derived from lipids. These changes occur through increased hepatic glucose production, glucose sparing by non-mammary tissues, increased glucose use by the mammary gland, increased hepatic glycogen mobilization, decreased lipogenesis, increased lipolysis, increased proteolysis, increased ketogenesis and increased use of ketone bodies (Bauman and Currie, 1980; Bell, 1995).

The changes of total body metabolism at the end of pregnancy and the beginning of lactation are regulated by altered concentrations of circulating hormones and altered tissue sensitivity to these hormones. The concept of these coordinated metabolic changes to support an altered physiological state is defined as 'homeorhesis' (Bauman and Currie, 1980). Other examples in which homeorhesis is of main importance, are prolonged fasting, growth and hibernation. Homeorhesis is regulated on a chronic basis, and takes place at multiple tissues and is partly mediated by altered responses to homeostatic signals. In contrast to the concept of homeorhesis, homeostasis is defined as a more acute regulation of metabolism to maintain constant conditions within a physiological state (Bauman and Currie, 1980; Bauman, 2000).

An overview of metabolic adaptations that occur at the level of lipid, carbohydrate and protein metabolism in the periparturient period of dairy cows is given in Table 1.

Table 1: Overview of metabolic adaptations that occur at the level of lipid, glucose and protein metabolism in the periparturient period of dairy cows (adapted from Ingvarsten, 2006 and Vernon, 2002).

	Metabolic change
Lipid metabolism	↓ lipogenesis ↑ lipolysis
Glucose metabolism	↑ use of fatty acids and ketone bodies by skeletal muscle ↓ use of glucose by skeletal muscle and adipose tissue ↑ gluconeogenesis
Protein metabolism	↑ uptake by uterus and mammary gland ↓ protein synthesis ↑ protein breakdown

The metabolic changes during the transition period of dairy cows are well described and regulated by different hormones. The most important hormones involved in the coordination of metabolism during late pregnancy and early lactation are given in Table 2.

Table 2: Hormones involved in the coordination of metabolism during late pregnancy and early lactation and their observed change in concentration (↑: increase; ↓: decrease; -: no change) during the periparturient period (adapted from Ingvarsten, 2006).

	Late pregnancy	Early lactation
Progesterone	↓	↓
Estrogen	↑	↓
Prolactin	↑	↓
Glucocorticoids	↑	—
Growth hormone	(↑)	↑
IGF-1	—	↓
Catecholamines	—	↑
Glucagon	—	↑
Insulin	—	↓

In the last days before delivery, important changes occur in the concentration of prolactin, progesterone, estrogen and glucocorticoids. While progesterone concentrations decrease rapidly, prolactin, estrogen and glucocorticoids increase to peak around calving and decrease to basal levels within few days post partum (Goff and Horst, 1997; Ingvarsten and Andersen, 2000). The changes of prolactin, progesterone, estrogen and glucocorticoids are mainly involved in the adaptation of the body towards parturition and the onset of milk production (lactogenesis) although they also modulate lipid, glucose and protein metabolism (Bell, 1995; Bell and Bauman, 1997). On the other hand, growth hormone, IGF-1, catecholamines, glucagon and insulin will change tissue metabolism in order to safeguard energetic

requirements of the most important tissues. Typically, growth hormone, glucagon and catecholamines are increased during early lactation while insulin and IGF-1 concentrations are low (Ingvarsen and Andersen, 2000). Especially the increase in GH is considered to be the driving factor for milk production. The increase in concentration of GH at the initiation of lactation in high producing dairy cows is appointed to the uncoupling of the GH-IGF axis. The normal IGF-1 production by the liver in response to GH is reduced due to a downregulation of growth hormone receptors in the liver, and IGF-1 concentrations will drop. Since IGF-1 inhibits GH production by the pituitary gland, the negative feedback loop is interrupted and GH production increases (Lucy et al., 2009; Roche et al., 2009). Growth hormone is responsible for a reduced insulin action at the level of the skeletal muscle and the adipose tissue, increased gluconeogenic activity of the liver and an increased catecholamine stimulated lipolytic activity of adipocytes (Etherton and Bauman, 1998; Bauman, 2000).

7. PRECIOUS BALANCE

Dramatic changes occur during the transition period of dairy cows, being the last three weeks before calving and the first three weeks of lactation (Drackley, 1999). Dairy cows walk the line between successful transitioning from pregnancy to lactation and unsuccessful transitioning with development of infectious and metabolic disorders compromising the profitability of a cow in the herd. Despite much progress is made in the knowledge of the intermediary metabolism of dairy cows during the transition period, 30 to 50 % of the dairy cows suffer from one or more disorder in the transition period (Leblanc, 2010). Recent studies indicate that 5 to 7 % of periparturient dairy cows suffer from clinical milk fever, 25 to 54 % suffer from subclinical hypocalcemia, 18 to 37 % suffer from postpartum metritis, 40 to 60 % suffer from ketonemia and 5 % suffer from abomasal displacement (Shaver, 1997; Duffield et al., 1998; Drillich et al., 2001; Reinhardt et al., 2011; McArt et al., 2012; Oetzel, 2013). However, there is a large variation in incidence of these metabolic and infectious diseases between herds. Different risk factors at the herd- and cow-level have been identified and allow veterinarians and dairy managers to optimize management, nutrition and environment of transition dairy cows (Cook and Nordlund, 2004; Mulligan et al., 2006; Bach et al., 2008; Leblanc, 2010).

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CHAPTER 2

Walking the line: the precious balance between health and disease in fresh cows with special reference to insulin resistance and overconditioning

Adapted from: De Koster J. and Opsomer G. 2012. Are modern dairy cows suffering from modern diseases? Vlaams Diergeneeskundig Tijdschrift. 81:71-80.

And: De Koster J. and Opsomer G. 2013. Insulin resistance in dairy cows. Veterinary Clinics of North America: Food Animal Practice. 29:299-322.

1. ARE MODERN DAIRY COWS SUFFERING FROM MODERN DISEASES?

Nowadays, dairy cows are capable of producing enormous amounts of milk. From 1951 till now, milk production has nearly quadrupled in the USA (based on the Crop Report of April 1, 1951 and the Milk Production Report of November 18, 2016 from the United States Department of Agriculture). This high level of milk production is mainly made possible by a long-time genetic selection towards higher milk production. However, the expression of this genetic potential is only fully achieved when the cows are managed under optimal conditions. Deviations from these optimal conditions easily result in an increased risk to develop health problems, especially during the immediate postpartum period.

Overconditioning at calving is one of the most important risk factors for dairy cows to develop a range of health problems during the transition period (Roche et al., 2009; Chebel and Ribeiro, 2016). The fat cow syndrome was first described by Morrow (1976) as a combination of metabolic, digestive, infectious and reproductive disorders that affects overconditioned periparturient dairy cows.

Currently, a very similar syndrome receives attention in human medicine mainly affecting obese people and rendering them more susceptible to a range of health problems, with cardiovascular disease and type 2 diabetes mellitus as the most important. The latter syndrome has been named the metabolic syndrome, and was formerly known as ‘the deadly quartet’ and ‘syndrome X’ (Cornier et al., 2008). The adipose tissue of these obese patients seems to play a central role in the pathophysiology of the syndrome by producing several bioactive molecules, also known as adipokines (Hutley and Prins, 2005).

The detrimental effects of excessive accumulation of body fat are not limited to cows and humans. Obesity has been assigned as an important risk factor for the development of non-communicable diseases in different species. Obese dogs and cats have a shorter life expectancy and are at increased risk to develop diabetes mellitus, neoplasia, cardio-respiratory, gastro-intestinal, orthopedic and urogenital disorders (German et al., 2010; Zoran, 2010). In horses, there is an important relationship between obesity and the development of insulin resistance and laminitis (Geor, 2008; Frank, 2009). Postpartum dysgalactia in sows has been associated with different risk factors, one of them being overconditioning at parturition (Papadopoulos et

al., 2010). In accordance with the human metabolic syndrome, it is supposed that the adipose tissue provokes a derailment of the normal physiological processes in obese animals, thereby rendering them more susceptible to different health problems.

By comparing the human metabolic syndrome with the fat cow syndrome, we might get a better understanding of the pathophysiology of the fat cow syndrome, which may lead to innovative insights into how to improve the management and treatment of modern dairy cows in the transition period.

2. THE HUMAN METABOLIC SYNDROME

2.1. Situation and importance

The increasing prevalence of obesity is an important health concern for the modern human population. The World Health Organization estimated that in 2008 at least 500 million adults were obese (body mass index or BMI > 30) whereas 1.5 billion adults were overweight (BMI > 25). Simultaneously, the prevalence of different obesity associated diseases, such as type 2 diabetes mellitus and cardiovascular disease, is increasing. A special term has been created to identify obese people who are at high risk to develop type 2 diabetes mellitus and cardiovascular disease. This term is called the metabolic syndrome (Cornier et al., 2008). The metabolic syndrome was defined by the International Diabetes Federation (IDF, 2006): ‘for a person to be defined as having the metabolic syndrome, he or she must have:

- central obesity (waist circumference ≥ 94 cm for European men and ≥ 80 cm for European women)
- plus two of the following four factors:
 - o raised triglyceride level (> 150 mg/dl),
 - o reduced HDL (high density lipoprotein) cholesterol (< 40 mg/dl for men and < 50 mg/dl for women),
 - o raised blood pressure (systolic blood pressure ≥ 130 mm Hg or diastolic blood pressure ≥ 85 mm Hg),
 - o raised fasting plasma glucose (≥ 100 mg/dl) or previously diagnosed type 2 diabetes mellitus.’

The IDF estimates that 20 to 25% of the adult population worldwide can be classified as suffering from the metabolic syndrome. Besides cardiovascular disease and type 2 diabetes mellitus, other conditions associated with obesity and the metabolic syndrome are non-alcoholic fatty liver disease, polycystic ovarian syndrome, obstructive sleep apnea, hypogonadism, microvascular disease, immune dysfunction and periodontitis (Marti et al., 2001; Cornier et al., 2008; Bullon et al., 2009).

At present, much attention is given to the role of the adipose tissue in these obesity associated diseases, with special emphasis on adipokines, non-esterified fatty acids (NEFA), metaflammation (= a form of chronic, low-level systemic inflammation, being linked to the metabolic syndrome) and abdominal obesity (Lafontan and Berlan, 2003; Despres and Lemieux, 2006; Hotamisligil, 2006; Cornier et al., 2008).

2.2. Adipokines

Research on the role of the adipose tissue in the pathogenesis of the metabolic syndrome has revealed that the adipose tissue is capable of secreting a wide range of different proteins, called adipokines (Pittas et al., 2004). Up till now, more than 50 different adipokines have been identified (Trayhurn and Wood, 2005). These adipokines act locally (autocrine effect) or are secreted in the peripheral circulation to have endocrine effects (Prins, 2002) (Table 1).

Table 1. List of the most important adipokines with their potential effects (Guerre-Millo, 2004; Meier and Gressner, 2004; Wozniak et al., 2009).

Adipokine	Potential effects
Leptin	↓ food intake, ↑ energy expenditure, ↑ insulin sensitivity, pro-inflammatory
Adiponectin	↑ insulin sensitivity, anti-inflammatory, anti-atherogenic
Resistin	pro-inflammatory, ↓ insulin sensitivity in rodents (effect in humans not clearly identified)
TNF (Tumor necrosis factor)	↓ insulin sensitivity, pro-inflammatory
IL6 (Interleukin 6)	↓ insulin sensitivity, pro-inflammatory

The adipose tissue of obese people, produces more pro-inflammatory adipokines (TNF, IL6, leptin, monocyte chemoattractant protein-1 or MCP-1) and less anti-inflammatory adipokines (adiponectin). The most important consequences of the altered secretion of adipokines, as seen

in obesity, are the induction of a pro-inflammatory state, cardiovascular damage and insulin resistance of the adipose tissue, the liver and the skeletal muscle (Cornier et al., 2008).

Structurally, some of the adipokines share similarities with pro-inflammatory cytokines. The globular fragment of adiponectin resembles the structure of TNF (Kershaw and Flier, 2004). Leptin shares structural similarity with IL6, IL12 and granulocyte colony-stimulating factor (Tilg and Moschen, 2006). Resistin belongs to a family of resistin-like molecules which are associated with inflammatory processes (Tilg and Moschen, 2006).

2.3. The pro-inflammatory state

Due to the altered production of adipokines, obese people are considered to be in a pro-inflammatory state with the adipose tissue itself as the primary site of inflammation (Gustafson et al., 2007). This chronic pro-inflammatory state is sometimes referred to as a 'metaflammation' or metabolically triggered inflammation (Hotamisligil, 2006). The adipose tissue becomes inflamed when an excessive amount of triglycerides are stored in the adipocytes. Enlarged adipocytes react to this stress by producing more pro-inflammatory adipokines and less anti-inflammatory adipokines. The latter results in an infiltration of macrophages in the adipose tissue, which contributes to the production and secretion of pro-inflammatory proteins (Cancello and Clement, 2006; Gustafson et al., 2007) (Figure 1).

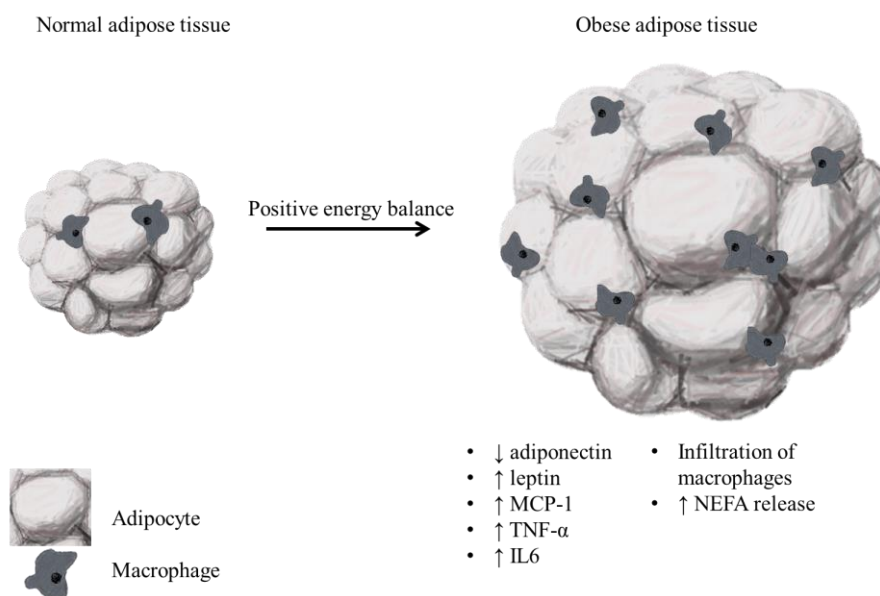


Figure 1. When the adipose tissue becomes 'obese', it gets infiltrated by macrophages. Additionally, the adipose tissue releases more NEFA and inflammatory adipokines whereas the secretion of anti-inflammatory adipokines (adiponectin) decreases (Gustafson et al., 2007).

In addition, some of the hypertrophic adipocytes become necrotic, which enhances a further infiltration of macrophages (Cinti et al., 2005). The increased concentration of circulating NEFA as typically observed during obesity, contributes to the production of pro-inflammatory adipokines by activating the inflammatory pathways in both adipocytes and macrophages (Shi et al., 2006). Interestingly, the pro-inflammatory state associated with obesity increases the susceptibility to various infections in obese people and animals (Dixit, 2008). In obese dogs, mortality due to canine distemper virus has been shown to be significantly increased (Bresnahan and Newberne, 1968), while obese mice display a six fold increase in mortality due to influenza virus infection (Smith et al., 2007). However, the exact mechanism by which obesity induces a pro-inflammatory impaired immune status, is not yet fully elucidated.

The aforementioned structural similarities between adipokines and inflammatory cytokines together with the presence of immune cells (macrophages and Kupffer cells) in metabolic important organs (adipose tissue and liver) demonstrate the importance of the link between metabolism and immunity (Hotamisligil, 2006). Evolutionary, the origin of this close relationship is situated in the fact that the immune cells, the liver and the adipose tissue have evolved from a common structure (Hotamisligil, 2006). Physiologically, metabolism and immune function need to be regulated in a coordinated way because in time of inflammatory events, the metabolism needs to adapt to support sufficient energy at the place of inflammation. In this situation, insulin resistance is favorable because this diverges energy away from anabolic processes (Hotamisligil, 2006). Nevertheless, the inflammation associated with obesity is chronic and therefore detrimental for both the metabolism and immune function (Hotamisligil, 2006).

2.4. Non-esterified fatty acids

Besides the altered production of adipokines, the obese body is also exposed to an elevated concentration of circulating NEFA (Lewis et al., 2002). The latter have direct and indirect effects on the health state of the obese person: indirectly by stimulating the production of adipokines, and directly by inducing an insulin resistant state of the liver (increased gluconeogenesis), the skeletal muscle (decreased glucose uptake) and the adipose tissue (decreased glucose uptake and decreased inhibition of lipolysis by insulin) (Van Epps Fung et

al., 1997; Lewis et al., 2002; Jensen, 2006a; Shi et al., 2006). Chronic exposure of the pancreas to high NEFA concentrations results in an impaired insulin secretion by the β -cells (Jensen, 2006a). These alterations contribute to the development of type 2 diabetes mellitus further harming the health state of obese patients (DeFronzo, 2004; Kahn et al., 2006).

2.5. Central or abdominal obesity

Basically, obese people can be divided into two groups based on the location of fat accumulation. There are people that accumulate fat subcutaneously, mainly around the hips and the lower part of the abdomen. This form of obesity is called peripheral obesity and people suffering from this type of fat accumulation are often referred to as ‘the pears’, as their overall body shape has taken the form of a pear. Other people however accumulate fat subcutaneously at the upper part of the abdomen and in the visceral depots (omentum, mesenterium). The latter form of obesity is called central or abdominal obesity, and people suffering from this type of fat accumulation are referred to as ‘the apples’ (Lafontan and Berlan, 2003).

This distinction is of clinical importance because especially the accumulation of visceral fat is associated with an increased risk for obesity associated diseases, whereas this is less the case for the accumulation of subcutaneous fat (Arner, 1998; Lafontan and Berlan, 2003). This is also the reason why in the definition of the metabolic syndrome as stated by the IDF (2006), waist circumference is included instead of the body mass index (BMI) as the former reflects much better the accumulation of fat in the abdomen. There are ethnic- and sex-specific cut off values for this parameter, indicating that the location where obese people deposit their excessive amounts of fat, is both gender as well as race dependent (IDF, 2006). It is generally known that men are more at risk to acquire the apple-like body shape when they become obese, while obese women are more likely to become pear-like (Arner, 1997).

There are some important functional differences between these two forms of obesity. The visceral adipose tissue has a higher lipolytic activity than the subcutaneous adipose tissue. This is caused by a higher lipolytic effect of catecholamines and a lower antilipolytic effect of insulin at the visceral fat cells. The resulting higher NEFA concentration in the vena porta can lead to the development of hepatic insulin resistance (Arner, 1998; Lafontan and Berlan, 2003;

Jensen, 2006b). Hepatic insulin resistance, characterized by an increased gluconeogenesis, is a major contributor to the development of type 2 diabetes mellitus (Scheen and Lefèbvre, 1996). Besides the higher lipolytic activity of the abdominal fat depots in abdominally obese persons, the production of adipokines is also different in the visceral adipocytes. In central obesity, the circulating concentration of adiponectin, an anti-inflammatory adipokine, is decreased whereas the expression and production of pro-inflammatory adipokines, like TNF and IL6, are elevated in comparison to peripherally obese persons (Despres and Lemieux, 2006). These factors in combination with the more direct contact between the visceral fat depots and the liver, generally renders abdominally obese patients more susceptible to severe health problems in comparison with peripherally obese people.

3. THE FAT COW SYNDROME

3.1. Definition

The fat cow syndrome was first described by Morrow (1976) as a combination of metabolic, digestive, infectious and reproductive disorders that affects the obese periparturient dairy cow. However, defining obesity in dairy cows is difficult. Assessment of body condition score (BCS) is a practical method to evaluate the energy reserve of a cow at a specific time point in the lactation cycle (Schröder and Staufenbiel, 2006). The BCS system as used in dairy cattle is based on the subjective visual and/or tactile evaluation of the amount of subcutaneously stored fat at the lumbar, sacral and tail region. A score is assigned based on the amount of fat stored: a low score indicating a low amount of fat (emaciation) and a high score indicating a high amount of fat (overconditioning). By comparing the BCS of one and the same cow at different points in time, it is possible to have an idea of the energy balance over that period in that specific cow (Schröder and Staufenbiel, 2006; Bewley and Schutz, 2008). There are different BCS scales: a 5 point scale is used in the USA, a 10 point scale is used in New Zealand, an 8 point scale is used in Australia, a 6 point scale is used in the United Kingdom and a 9 point scale is used in Denmark (Bewley and Schutz, 2008; Roche et al., 2009). In the present manuscript, BCS will refer to the 5 point scale from the USA as described by Edmonson et al. (1989), using 5 points (1 = severe underconditioned, 5 = severe overconditioned) with 0.25 unit increments. It was estimated that a change of one BCS point equals approximately 50 kg

of total body fat (Schröder and Staufenbiel, 2006). Using this scale, a BCS of 3.5 can be interpreted as high for a cow in peak lactation while it can be considered normal for a dry cow, meaning that the stage in lactation of a cow has an important influence on the optimal BCS for that cow (Schröder and Staufenbiel, 2006). Normally, BCS drops after calving to reach a nadir at 40 to 100 DIM and increases gradually thereafter (Figure 2).

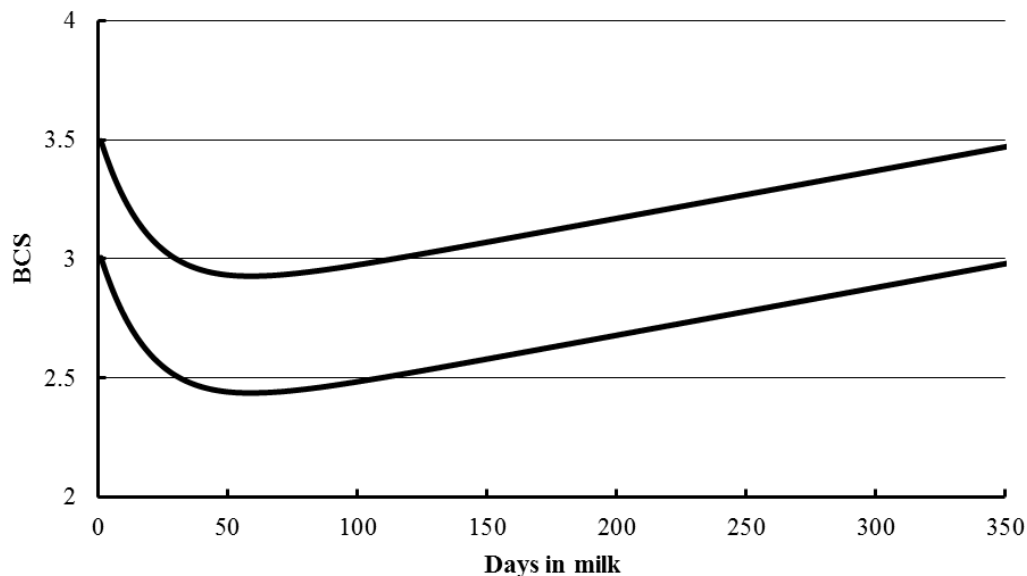


Figure 2: Profile of the optimal range of BCS during the lactation. Adapted from Chagas et al. (2007) and Roche et al. (2009).

During a state of negative energy balance, cows lose approximately 1 kg of body fat per day (Vernon and Pond, 1997). Ideally, cows should not lose more than 0.5 to 1.0 units of BCS (or 50 kg body fat) during the postpartum period and cows should not gain nor lose BCS during the dry period (Ingvarsen, 2006; Roche et al., 2009). Overconditioning in dairy cows is mainly a problem during the transition period. Roche et al. (2013) stated that the ideal BCS at calving is 3.0 for cows while heifers can be slightly fatter (ideal BCS at calving 3.25). Nevertheless, it is generally accepted that a dairy cow during the dry period should have a BCS of 3.0 to 3.5 (Chagas et al., 2007; Roche et al., 2009). As a consequence, it can be assumed that cows with a higher BCS during the dry period (≥ 3.75) are overconditioned. Despite the fact that Schröder and Staufenbiel (2006) defined obese cows as cows with a BCS of 5.0, there is no general consensus regarding the definition of obesity in dairy cows. So in the present manuscript, we will talk about overconditioned or fat dairy cows and avoid to use the term obesity in dairy cows.

3.2. Importance of the fat cow syndrome

Dairy cows become overconditioned when energy intake exceeds energy output. Typically, this occurs in cows with a low level of milk production and/or in cows with a long interval from calving to conception and/or in cows that are overfed during the dry period (Schröder and Staufenbiel, 2006; Chebel and Ribeiro, 2016). Overconditioning at calving and excessive BCS loss in the periparturient period are strongly related to each other (Roche et al., 2009; Chebel and Ribeiro, 2016) and both conditions are important risk factors for different disorders (Table 2).

Table 2: Odds ratios (OR) for overconditioning with different transition problems

Disease	BCS	OR	Reference
Reproductive diseases ^a	≥ 4.0	2.8	(Gearhart et al., 1990) ^b
Lameness	≥ 4.0	7	(Gearhart et al., 1990) ^b
Milk fever	≥ 4.0	3.3	(Heuer et al., 1999) ^c
LDA	+ 1 unit BCS	2.405	(Cameron et al., 1998) ^d
Ketosis	4.0 - 4.25	2.3	(Gillund et al., 2001) ^e
Ketosis	> 4.5	2.8	(Gillund et al., 2001) ^e
Ketosis	≥ 4.0	1.58	(Duffield et al., 1998) ^f
Ketosis	+ 1 unit BCS	2.2	(Markusfeld et al., 1997) ^g

^a Reproductive diseases including dystocia, retained placenta, cystic ovaries, metritis, endometritis, pyometra and abortion.

^b OR for dry off BCS in comparison with cows with BCS of 2.25 to 3.75.

^c OR for first BCS during lactation in comparison with cows with BCS of 2.25 to 3.75.

^d OR obtained from a multivariable logistic model for BCS during the dry period.

^e OR for BCS at calving in comparison with cows with BCS 3.0 to 3.25.

^f OR for dry period BCS in comparison with cows with BCS of 3.25 to 3.75.

^g OR obtained from a multivariable logistic model for BCS at calving.

Despite increased odds for different disorders in overconditioned cows as presented in Table 2, this relationship is not a consistent finding for all published studies with fat cows. The reason for this is that in many studies, a limited number of overconditioned cows was included or that the relationships were confounded by changes of BCS during the dry period which may influence disease incidence as well (Waltner et al., 1993; Bewley and Schutz, 2008).

Overconditioned cows have a reduced appetite and therefore a lower prepartum dry matter intake (DMI) and a slower increase in postpartum DMI (Grummer et al., 2004). As a consequence of this reduced DMI, overconditioned cows start the lactation in a more severe negative energy balance (NEB) than their normal conditioned counterparts (Grummer et al., 2004). The latter contributes to the increased fat mobilization observed in overconditioned cows in the periparturient period (Roche et al., 2009).

3.3. Lipid metabolism

The change in BCS throughout lactation is a reflection of the change in lipid metabolism during lactation. Lipogenesis and lipolysis are continuous processes occurring simultaneously within the adipocytes. The relative balance between both processes determines if there is net accumulation or net mobilization of triglycerides (Vernon, 2002). At the end of lactation and during the dry period, adipose tissue is storing energy as triglycerides in adipocytes (increased lipogenesis and decreased lipolysis) which are released during the negative energy balance in early lactation (increased lipolysis and decreased lipogenesis) (Vernon and Pond, 1997; Roche et al., 2009). In early lactation, different factors (eg. high growth hormone and glucagon, low insulin) favor the lipolytic pathway, while at the end of lactation different factors (eg. high insulin, low growth hormone and glucagon) favor the lipogenic pathway (Ingvarsen, 2006). Lipogenesis occurs within the cytoplasm of adipocytes, epithelial cells of the mammary gland and to a lesser extent in hepatocytes and myocytes. Three molecules of fatty acids are esterified with one molecule of glycerol resulting in the formation of one molecule triacylglycerol (TAG). Fatty acids are synthesized *de novo* or are taken up from the bloodstream. *De novo* synthesis of fatty acids is regulated by the activity of 2 enzymes: acetyl CoA carboxylase (ACC) and fatty acid synthetase (FAS). For the *de novo* synthesis of fatty acids, ruminants preferably use acetate as substrate instead of glucose. Uptake of circulating fatty acids occurs via endothelial lipoprotein lipase (LPL) (Figure 3) (Vernon, 2002; Roche et al., 2009).

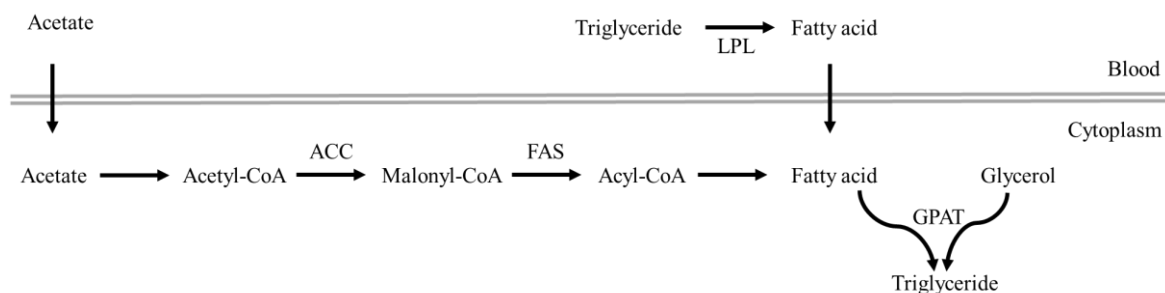


Figure 3: Overview of the major lipogenic pathways. (ACC, acetyl-coA carboxylase; FAS, fatty acid synthetase; LPL, lipoprotein lipase; GPAT, glycerol-3-phosphate acyl transferase). Adapted from Vernon (2002) and Ingvarsen (2006).

Lipolysis is mediated by the consecutive action of 3 enzymes: adipose triglyceride lipase (ATGL), HSL (hormone sensitive lipase) and MGL (monoacylglycerol lipase). One molecule of triacylglycerol is broken down into three fatty acid molecules and one molecule of glycerol. Fatty acids are released in the bloodstream or are re-esterified in the lipogenic pathway. The hydrolysis of TAG is initiated by ATGL, next HSL hydrolyses diacylglycerol with the formation of monoacylglycerol. Monoacylglycerol lipase is responsible for the final step of the lipolytic pathway that produces the final fatty acid molecule and glycerol (Figure 4) (Jaworski et al., 2007; Zechner et al., 2009).

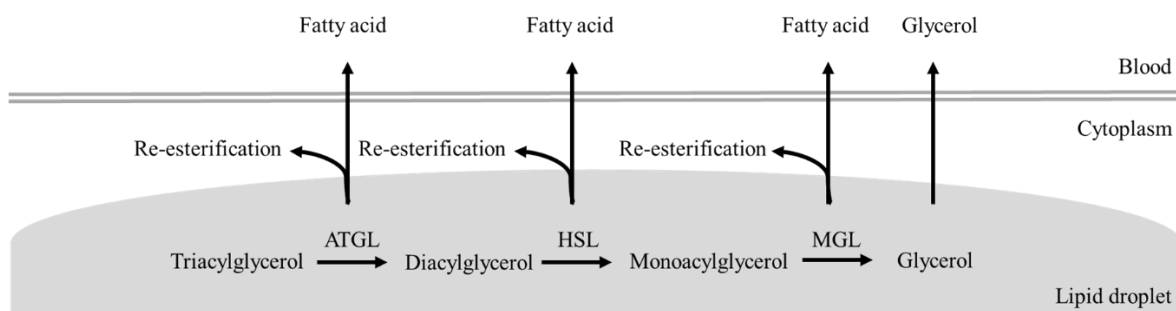


Figure 4: Overview of the major lipolytic pathways. (ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; MGL, monoacylglycerol lipase) (Adapted from Lafontan and Langin (2009) and Zechner et al. (2009).

Lipolysis is stimulated via β -adrenergic receptors which activate PKA (protein kinase A) by increasing the intracellular concentration of cAMP. Catecholamines are the major hormones to stimulate lipolysis and reach the adipose tissue via circulation (adrenalin) or via sympathetic innervation (noradrenalin). Lipolysis is inhibited by insulin which activates PDE-3B (phosphodiesterase 3B) with subsequent degradation of cAMP and decreased activation of PKA. PKA phosphorylates different target molecules, among which HSL is the most important one. Phosphorylation of HSL increases its lipolytic activity. An additional factor that is phosphorylated upon stimulation of β -adrenergic receptors is perilipin. Perilipin is a protein that is localized at the surface of the lipid droplet. Upon phosphorylation, perilipin is translocated from the lipid droplet, rendering the surface of the lipid droplet accessible to HSL. Another protein, CGI-58 (comparative gene identification 58), is bound to unphosphorylated perilipin and detaches when perilipin is phosphorylated. Unbound CGI-58 stimulates the activity of ATGL to hydrolyze TAG (Duncan et al., 2007; Jaworski et al., 2007; Lafontan and Langin, 2009; Zechner et al., 2009).

The adipose tissue of overfed and overconditioned cows tends to be more sensitive for lipolytic and less sensitive for antilipolytic stimuli (Rukkwamsuk et al., 1998). The combination of a more severe NEB and a higher lipolytic activity of the adipose tissue results in an excessive mobilization of NEFA in the overconditioned periparturient dairy cow. A large proportion of the circulating NEFA are taken up by the liver and are metabolized in three ways: complete oxidation in mitochondria or peroxisomes, partial or incomplete oxidation in mitochondria (ketogenesis) or re-esterification in the cytosol (Figure 5) (Herd, 2000; Drackley et al., 2001b; Drackley et al., 2005; Roche et al., 2009).

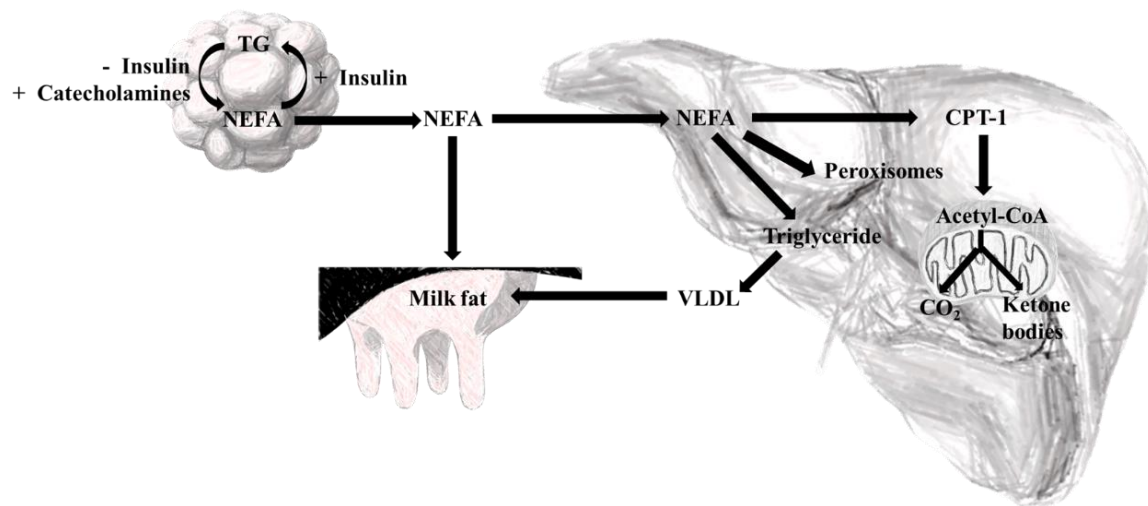


Figure 5: Metabolism of NEFA in the periparturient dairy cow (adapted from Drackley (1999) and Roche et al. (2009)).

First, NEFA can be completely oxidized in the Krebs cycle with the production of carbon dioxide and energy (ATP). For this reaction, the hepatocytes need oxaloacetate. In periods of NEB and high glucose requirements (late pregnancy, lactation), the precursor molecules for this oxaloacetate are limited and the available oxaloacetate is preferentially used as substrate for gluconeogenesis.

A part of the NEFA are oxidized in the peroxisomes. Peroxisomal β -oxidation of NEFA serves as an overflow mechanism if NEFA supply to the liver is excessive. Peroxisomal oxidation of NEFA is energetically inefficient and results in the production of hydrogen peroxide and heat. Some of the mobilized NEFA are not completely oxidized and are redirected to other metabolic

pathways (Bell and Bauman, 1997; Rukkwamsuk et al., 1999a; Bossaert et al., 2008b; Roche et al., 2009).

Secondly, incomplete oxidation of NEFA will increase the intracellular levels of acetyl coenzyme A. This acetyl coenzyme A is the precursor for the ketone bodies, acetoacetate, β -hydroxybutyrate and aceton (Herdt, 2000; Bossaert et al., 2008b). Excessive production of ketone bodies results in the development of (sub)clinical ketosis.

Thirdly, the hepatocytes can re-esterify NEFA with the production of triglycerides. These triglycerides are secreted in the blood in very low density lipoproteins (VLDL), which consist of apoprotein B, triglycerides, cholesterol, cholesterol-esters and phospholipids. When the increased hepatic production of triglycerides exceeds the hepatic synthesis of VLDL, triglycerides are stored in the parenchyma of the liver, which leads to hepatic lipidosis or fatty liver (Rukkwamsuk et al., 1999a; Herdt, 2000; Bossaert et al., 2008a).

This is the well-known story of how a fat cow develops fatty liver and ketosis. With the current knowledge of the role of the adipose tissue in the development of obesity associated diseases in human medicine, we can no longer ignore a possible role of the endocrine function of the adipose tissue in the development of the fat cow syndrome.

3.4. Comparison between the human metabolic syndrome and the fat cow syndrome

When comparing the human metabolic syndrome with the fat cow syndrome, it is clear that there are a lot of similarities. Overconditioned cows are thought to be insulin resistant (McCann and Reimers, 1985; Holtenius and Holtenius, 2007), the adipose tissue of dairy cows is also capable of producing different adipokines (Ingvarsen and Boisclair, 2001; Komatsu et al., 2003; Komatsu et al., 2005; Lemor et al., 2009; Mukesh et al., 2010; Sadri et al., 2010), the disease susceptibility of dairy cows is associated with a pro-inflammatory state (Ohtsuka et al., 2001; Ametaj et al., 2005; Bertoni et al., 2008; Bradford et al., 2009), the immunity of overconditioned cows is attenuated (Lacetera et al., 2005) and overconditioned dairy cows are overall more susceptible to a variety of diseases (Morrow, 1976; Roche et al., 2009).

Apart from these similarities, there are some important differences between both syndromes. In human medicine, obesity is associated with insulin resistance (normoglycemia and secondary hyperinsulinemia) or type 2 diabetes mellitus (hyperglycemia). Due to the special carbohydrate metabolism in lactating dairy cows, the glucose and insulin concentrations during early lactation are low due to the high loss of glucose through the production of milk (Table 3, Figure 6 and 7) (Herdt, 2000).

Table 3. Comparison of the human metabolic syndrome and the fat cow syndrome (↑ = increased; ↓ = decreased; ? = unknown).

Property	Human metabolic syndrome	Fat cow syndrome	Reference
Disease susceptibility	↑	↑	(Morrow, 1976; Cornier et al., 2008)
Adipokine production	↑ (except ↓ adiponectin)	? (except ↑ leptin and TNF)	(Ingvarsen and Boisclair, 2001; O'Boyle et al., 2006; Cornier et al., 2008)
Inflammation	↑	↑	(Ametaj et al., 2005; O'Boyle et al., 2006; Gustafson et al., 2007)
Immune function	↓	↓	(Lacetera et al., 2005; Dixit, 2008)
Insulin sensitivity	↓	? or ↓	(McCann and Reimers, 1985; Cornier et al., 2008)
Basal insulin concentration	Normal or ↑	Normal (low)	(Herdt, 2000; DeFronzo, 2004)
Glucose stimulated insulin secretion	Normal, ↑ or ↓	?	(DeFronzo, 2004)
Glucose concentration	Normal or ↑	Normal (low)	(Herdt, 2000; DeFronzo, 2004)
Obesity	Visceral	?	(Despres and Lemieux, 2006)

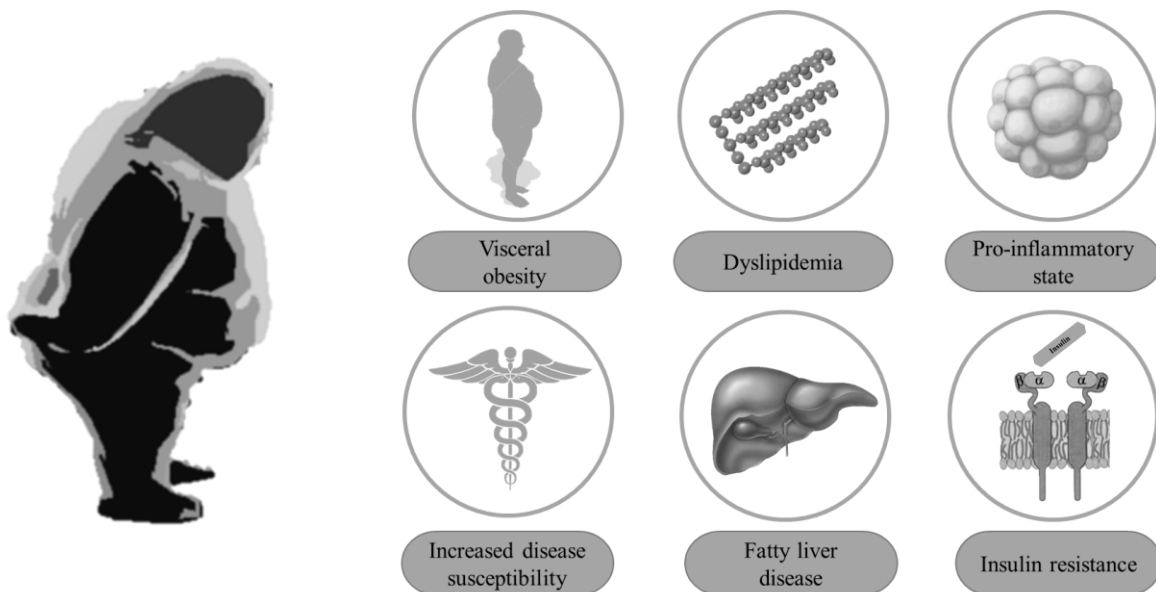


Figure 6: Characteristics of the human metabolic syndrome

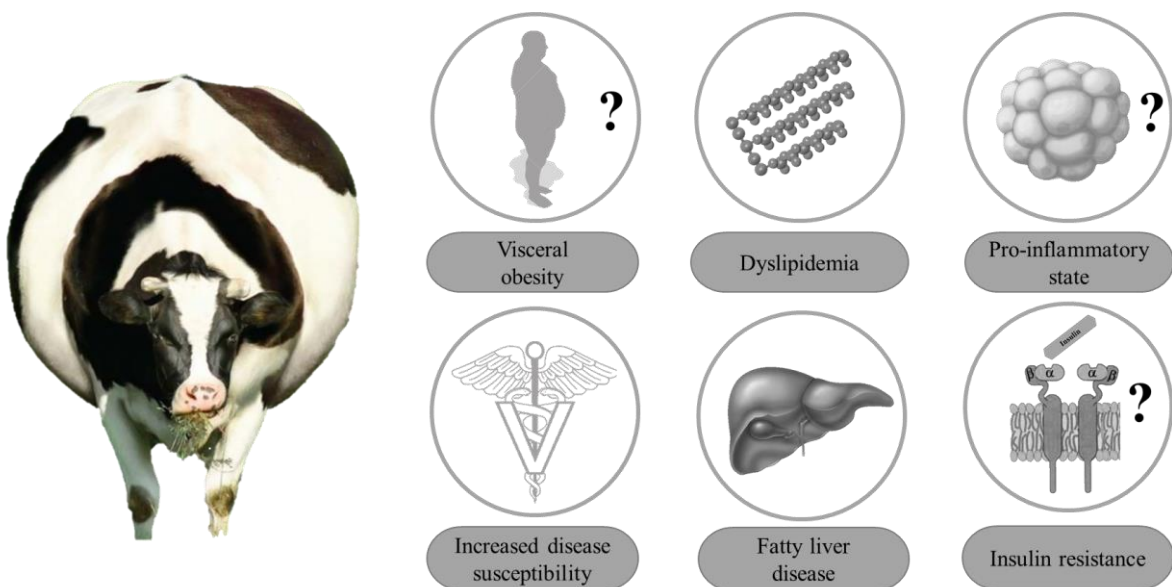


Figure 7: Characteristics of the fat cow syndrome

3.5. Nonesterified fatty acids in dairy cows

The physiological and pathological role of NEFA in dairy cattle are well-studied. Triglycerides are the most important energy reserve and are stored in lipid droplets in adipocytes. During NEB, triglycerides are mobilized as NEFA (Herdt, 2000; Roche et al., 2009). As long as the NEFA release from the adipose tissue is limited, this can be seen as favorable since NEFA can directly or indirectly (as ketone bodies) be used as energy substrate in different tissues, thereby sparing glucose for milk production. However, excessive fat mobilization results in an overload of NEFA with a negative impact on production, reproduction and insulin sensitivity (Pires et al., 2007b; Bossaert et al., 2008c).

Especially in ruminants, an overload of NEFA easily surpasses the liver's capacity to produce apoprotein B and VLDL. This leads to an increased amount of triglycerides being accumulated in the hepatocytes (Gruffat et al., 1996). This fatty infiltration of the liver impairs the hepatic metabolism, which results in a reduced activity of gluconeogenic enzymes (Rukkwamsuk et al., 1999b). Since in cattle the liver is the primary site of glucose production, the reduced gluconeogenic activity results in a decreased glucose production and a reduced milk production.

Research at our department furthermore revealed that high circulating concentrations of NEFA may reach the follicular fluid in the ovary and have a detrimental effect on oocyte quality which results in poor in vitro results regarding maturation, fertilization, cleavage rate and blastocyst yield (Leroy et al., 2005).

As in humans, also in dairy cows there seem to be individuals in which the secretion of insulin by the pancreas is seriously compromised. In a study in which cows, previously diagnosed with cystic ovarian disease, were submitted to an intravenous glucose tolerance test, Opsomer et al. (1999) found 3 cystic cows that did not react with an increased insulin secretion following the administration of an intravenous glucose bolus. In none of the matched control cows, a similar absence of insulin secretion could be detected. A similar absence of insulin secretion following the administration of a glucose bolus in dairy cows was demonstrated by Hove (1978) in ketonemic and starved cows. Bossaert et al. (2008c) have demonstrated that the insulin secretion following the administration of an intravenous glucose bolus is negatively

associated with the level of NEFA that cows had experienced in the periparturient period. This leads us to conclude that in dairy cows as well as in humans, there are individuals that have susceptible β -cells whose function becomes seriously depressed by chronically elevated NEFA levels. As differences in pancreatic insulin secretion following an intravenous glucose bolus have already been demonstrated in neonatal calves (Bossaert et al., 2009), this β -cell susceptibility has been hypothesized to have a(n) (epi)genetic background.

3.6. Adipokines in dairy cows

The altered production of adipokines by the visceral fat depots seems to be the key factor to develop health problems in abdominally obese persons. Research towards gene expression in adipose tissue of dairy cows has confirmed the role of the adipose tissue as an endocrine organ. Different studies have demonstrated an expression of mRNA for TNF, IL6, MCP-1, leptin, adiponectin, visfatin and resistin in the adipose tissue of dairy cows (Ingvarsen and Boisclair, 2001; Komatsu et al., 2003; Komatsu et al., 2005; Lemor et al., 2009; Mukesh et al., 2010; Sadri et al., 2010). To the best of our knowledge, the exact function of most of the adipokines in dairy cows are unknown, except for leptin. Leptin is an important regulator of feed intake and influences the adaptational mechanisms during the transition period. However, there are also indications that leptin may influence the reproductive and immune systems (Ingvarsen and Boisclair, 2001). For leptin, it is known that the plasma concentration in dairy cows is influenced by BCS and energy balance with a higher plasma concentration in cows with a high BCS and a lower plasma concentration during early lactation (Ingvarsen and Boisclair, 2001; Meikle et al., 2004). For adiponectin, a possible role in the adaptational mechanism is expected because of the increase in plasma concentration during the first weeks of lactation. It reaches a maximum value at four weeks post partum. Subsequently, the value declines and remains stable from the fifth till the eleventh week post partum (Giesy et al., 2012; Mielenz et al., 2013).

For TNF, it has been demonstrated that cows with a BCS > 3.5 tend to have a higher plasma TNF concentration than cows with a normal BCS (BCS 2.5-2.7) during mid-lactation (O'Boyle et al., 2006). Additionally, increased serum TNF concentrations have been associated with insulin resistance and the development of fatty liver (Ohtsuka et al., 2001; Bradford et al., 2009).

3.7. The pro-inflammatory state in dairy cows

Inflammatory reactions are characterized by an increased hepatic production of positive acute phase proteins (haptoglobin, C-reactive protein, serum amyloid A, ceruloplasmin) and a decreased hepatic production of negative acute phase proteins (albumin, lipoproteins, retinol-binding protein) (Bertoni and Trevisi, 2013).

A recent study in dairy cows has demonstrated a negative correlation between sustained low plasma concentration of negative acute phase proteins during lactation and health and fertility problems (Bertoni et al., 2008). The cows with chronically low concentrations of negative acute phase proteins, as measured by the average plasma concentration of albumin, cholesterol and retinol-binding protein at 7, 14 and 28 days in milk (DIM), had also the highest plasma concentration of haptoglobin, a positive acute phase protein. The cows with chronically low concentrations of negative acute phase proteins had a higher frequency of health problems (dystocia, milk fever, retained placenta, ketosis, lameness, mastitis) and fertility problems (Bertoni et al., 2008). Other studies confirm these observations by demonstrating a strong positive correlation between positive acute phase proteins and cytokines and the development of fatty liver post partum (Ohtsuka et al., 2001; Ametaj et al., 2005; Bradford et al., 2009). These data suggest a role for chronic low-grade inflammation in several diseases that occur during the transition period.

Typically, the increase in concentration of positive acute phase proteins occurs very quickly after an inflammatory stimulus but normal levels are quickly restored after resolving the inflammatory stimulus. On the other hand, the change in concentration of the negative acute phase proteins occurs slower but is longer in duration. Therefore, Bertoni et al. (2008) and Trevisi et al. (2012) use the concentration of negative acute phase proteins in their calculation of the liver activity index or the liver functionality index, to reflect changes of the inflammatory state of dairy cows over a longer time period.

3.8. Immune function and overconditioning in dairy cows

The association between overconditioning and the elevated incidence of infectious diseases has been explained by an impaired immune function in overconditioned cows. In the periparturient period, it is observed that the lymphocyte function is attenuated in overconditioned cows

(Lacetera et al., 2005). Moreover, overconditioned cows have higher circulating concentrations of reactive oxygen metabolites and lower circulating concentrations of antioxidants (Bernabucci et al., 2005). This increased oxidative stress may be an important cause of an attenuated immune function (Sordillo and Aitken, 2009).

3.9. Body condition score and abdominal fat accumulation in dairy cows

In dairy cows, body condition scoring (BCS) and the ultrasonographic measurement of back fat thickness are practical methods to describe the energy reserve at a specific point in time in the lactation cycle (Schröder and Staufenbiel, 2006). The total amount of fat stored in the body of dairy cows can be divided into different fat depots dependent on the localization. The largest amount of body fat can be found intermuscular (50%), followed by subcutaneous fat (15 - 20%), omental fat (10%), perirenal-retroperitoneal fat (10%), mesenteric fat (7% - 8%) and fat stored in other parts of the body (Butler-Hogg et al., 1985).

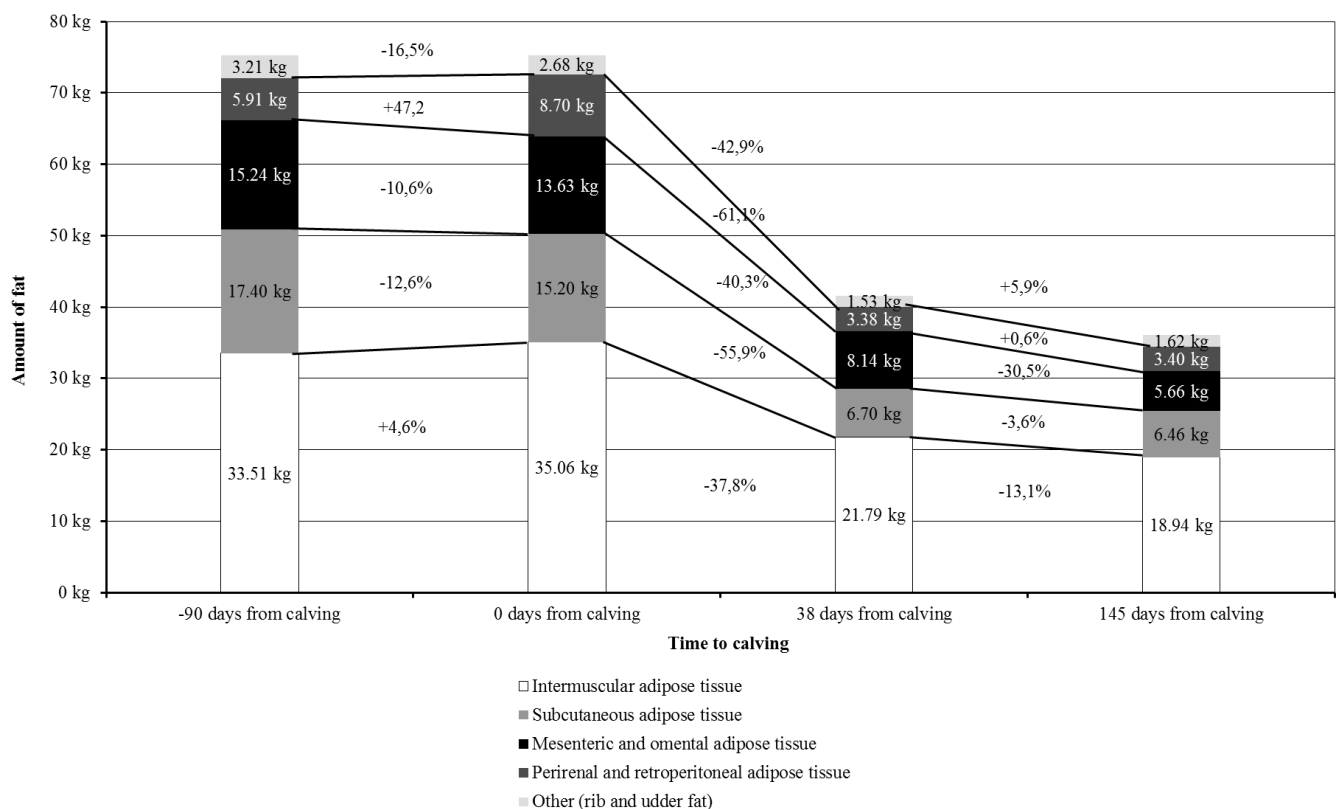


Figure 8. Average amount (in kg) of fat in the different fat depots of dairy cows slaughtered at different stages in the lactation cycle (four cows slaughtered per stage). Change (in %) of the size of the different fat depots relative to the previous point in time of each individual fat depot. Adapted from Butler-Hogg et al. (1985).

Hence, by assessing the BCS, only 15-20% of the total body fat, being the subcutaneous fat, is evaluated. There is a positive correlation between the BCS, the total amount of body fat and the amount of fat stored in the individual fat depots. Nevertheless, not all fat depots are mobilized or replenished at the same time at the same magnitude (Butler-Hogg et al., 1985). During early lactation in dairy cows, relatively more fat is mobilized from the subcutaneous and perirenal-retroperitoneal fat depots than from the intermuscular, omental and mesenteric fat depots (Butler-Hogg et al., 1985) (Figure 8).

In a recent study, nonpregnant nonlactating Holstein cows were either overfed energy or fed a controlled energy diet for a period of 8 weeks (comparable to the length of the dry period). The BCS of both groups of cows increased during the study but there was no difference in BCS between both groups. Interestingly, the overfed group had significantly greater amounts of omental, mesenteric and perirenal fat compared with the normal fed cows, so the overfed cows preferentially accumulated fat in the internal fat depots (Drackley et al., 2014).

This implies that a cow can have a normal body condition score even though she possesses a large amount of 'invisible' abdominal fat stored in the mesenterium and the omentum (Van Eetvelde et al., 2011). Additionally, dairy cows seem to have a genetic tendency to accumulate more fat in the intra-abdominal depot and less fat in the subcutaneous depot (Wright and Russel, 1984). In human medicine, the accumulation of fat in the abdomen is linked to an increased disease susceptibility. Whether the same holds true for dairy cows remains an open question.

3.10. Insulin resistance in dairy cows

3.10.1. Definition of insulin resistance

Insulin resistance is defined as a state where a normal concentration of insulin induces a decreased biological response in the insulin-sensitive tissues (Kahn, 1978). Insulin resistance can furthermore be subdivided based on two distinct features: insulin sensitivity and insulin responsiveness (Figure 9). The maximal effect of insulin determines the insulin responsiveness. The concentration of insulin needed to elicit the half-maximal response determines the insulin sensitivity (Kahn, 1978).

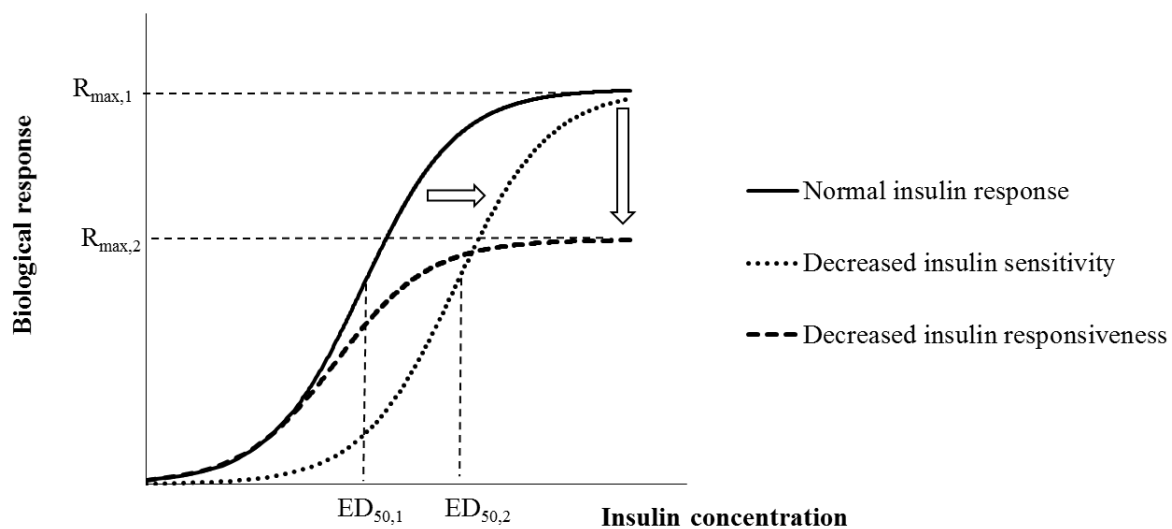


Figure 9: Graphical representation of the difference between insulin sensitivity and insulin responsiveness. The normal insulin response is characterized by a maximal biological effect ($R_{\max,1}$) and an insulin concentration to elicit a half maximal effect ($ED_{50,1}$). A decreased insulin sensitivity is visualized by a right shift of the normal curve and characterized by a normal maximal biological effect ($R_{\max,1}$), while an increased insulin concentration is needed to elicit half of the maximal effect ($ED_{50,2}$). A decreased insulin responsiveness is visualized by a down shift of the normal curve and is characterized by a decreased maximal biological effect ($R_{\max,2}$) while a normal insulin concentration is needed to elicit a half maximal effect ($ED_{50,1}$). Adapted from Kahn (1978).

Insulin resistance can hence be attributed to a decrease in insulin responsiveness (a downward shift of the insulin dose-response curve), a decrease in insulin sensitivity (a rightward shift of the insulin dose-response curve) or a combination of both (Kahn, 1978; Muniyappa et al., 2008). Insulin resistance can be specific for certain tissues and for certain biological processes within these tissues (Kahn, 1978; Bauman, 2000).

3.10.2. Insulin secretion and diabetes mellitus

A further necessary distinction needs to be made between insulin resistance and deficient insulin secretion. Insulin resistance is determined by the response of the insulin sensitive tissues to a normal concentration of insulin. Insulin secretion is determined by the secretory capacity of the pancreas in response to a factor that stimulates insulin secretion. Deficient insulin secretion does not entail an altered state of insulin resistance. The best way to explain this is by observing the difference between type 1 and type 2 diabetes in human medicine. Type 1 diabetes is caused by a destruction of the β cells of the pancreas resulting in an absolute insulin deficiency. Patients with type 1 diabetes have no insulin secretion but can have normal insulin sensitivity and responsiveness of their tissues (American Diabetes Association, 2004).

The most common form of type 2 diabetes is caused by a combination of insulin resistance and a relative deficiency of insulin secretion. In the early phase of the pathophysiology of type 2 diabetes mellitus, insulin resistance occurs but the pancreas compensates for this by increasing the secretion of insulin, giving rise to a secondary hyperinsulinemia. If the insulin resistance is not ameliorated, the pancreas may become exhausted, which may lead to β cells failing to compensate for the increased insulin resistance. Relative to the degree of insulin resistance, the pancreas cannot produce enough insulin (relative insulin deficiency) and levels of blood glucose rise, thus type 2 diabetes develops (Kahn, 2003; American Diabetes Association, 2004; DeFronzo, 2004). Insulin secretion in type 2 diabetes can be normal but is insufficient to compensate for the higher degree of peripheral insulin resistance.

Diabetes mellitus has been described in young cattle and dairy cows (Taniyama et al., 1993; Nazifi et al., 2004). The role of insulin resistance in the development of diabetes mellitus in cattle is unknown, but diabetes mellitus is considered to be a rare endocrine disease in dairy cows owing to its irreversible nature. By contrast, the insulin-secretory capacity of the pancreas seems to be influenced by NEFA in dairy cows. Bossaert et al. (2008c) demonstrated a negative impact of chronically elevated NEFA levels on the insulin secretory capacity of the pancreas. Similarly, lower insulin secretion following an intravenous glucose bolus has been demonstrated in ketonemic and starved cows (Hove, 1978; Kerestes et al., 2009; Schoenberg et al., 2012) and in cows suffering from cystic ovarian disease (Opsomer et al., 1999).

3.10.3. Effect of insulin resistance at different tissues

Insulin elicits different effects on the carbohydrate, lipid and protein metabolism of different insulin sensitive tissues (Table 4). Besides these effects on the glucose, amino acid and lipid metabolism, insulin has some additional effects on several tissues throughout the body. In general, insulin is known to stimulate cell proliferation and differentiation (DeFronzo, 2004). In the ovary, insulin stimulates steroidogenesis and the proliferation of granulosa cells and hence influences follicular growth (Bossaert et al., 2010; Sinclair, 2010). Lower peripheral insulin levels have been associated with the development of ovarian cysts in postpartum dairy cows (Vanholder et al., 2005).

Table 4: Overview of the effects of insulin on different metabolic pathways in the different insulin sensitive tissues.

Tissue	Metabolic pathway	Insulin effect	Reference
Liver	glycogenesis	stimulation	(Brockman and Laarveld, 1986)
	ketogenesis	suppression	(Brockman and Laarveld, 1986)
	TAG synthesis	stimulation	(Andersen et al., 2002)
	gluconeogenesis	suppression	(Brockman and Laarveld, 1986)
	glycogenolysis	suppression	(Brockman and Laarveld, 1986)
	glycolysis	stimulation	(Hayirli, 2006)
	protein synthesis	stimulation	(Sjaastad et al., 2010)
	protein degradation	suppression	(Sjaastad et al., 2010)
Skeletal muscle	glucose uptake	stimulation	(Brockman and Laarveld, 1986)
	ketone body use	stimulation	(Brockman and Laarveld, 1986)
	protein synthesis	stimulation	(Brockman and Laarveld, 1986)
	protein degradation	suppression	(Brockman and Laarveld, 1986)
	glycolysis	stimulation	(Hayirli, 2006)
	glycogenolysis	suppression	(Hayirli, 2006)
	glycogenesis	stimulation	(Hayirli, 2006)
Adipose tissue	lipolysis	suppression	(Brockman and Laarveld, 1986)
	lipogenesis	stimulation	(Brockman and Laarveld, 1986)
	glucose uptake	stimulation	(Brockman and Laarveld, 1986)

As previously mentioned, the development of insulin resistance may be specific for a certain metabolic pathway in a certain tissue. For dairy cows, the most important pathways that have an altered insulin response during the transition period are: glucose uptake by the skeletal muscle and the adipose tissue, lipogenesis and lipolysis in adipose tissue, gluconeogenesis in the liver, and protein metabolism of skeletal muscle. The role of insulin and insulin resistance in the adaptation of these metabolic pathways during the transition period are further explored.

3.10.4. Insulin-stimulated glucose uptake by the skeletal muscle and the adipose tissue

General aspects of insulin-stimulated glucose uptake

Insulin-stimulated glucose uptake is probably the most intensively studied metabolic pathway of insulin in dairy cows. Based on the effect of insulin on this pathway, conclusions are drawn to other metabolic pathways which may give rise to incorrect or at least insufficiently substantiated conclusions. In humans, skeletal muscles account for 80% of the insulin stimulated whole body glucose uptake, while adipose tissues contribute to only 5% (DeFronzo, 2004). In dairy cows, where acetate is the major precursor for triglyceride synthesis, the skeletal muscles account for the majority of the insulin stimulated whole body glucose uptake (Duhlmeier et al., 2005). Therefore, glucose uptake in adipose tissue is limited in dairy cows (Brockman and Laarveld, 1986). The fact that dairy cows are in a lactating and/or pregnant state means that a considerable amount of glucose is taken up by the pregnant uterus or the lactating udder. One should consider this carefully when comparing glucose uptake experiments in cows at different stages of lactation or in cows with a different level of milk production.

Insulin resistance for glucose uptake by skeletal muscles and adipose tissues is a well known feature in pregnant women, obese people and people suffering from type 2 or gestational diabetes (Stanley et al., 1998; Stolic et al., 2002; DeFronzo, 2004; Barbour et al., 2007; Cornier et al., 2008). Some of these metabolic states in humans show remarkable similarities with insulin resistance in cattle (De Koster and Opsomer, 2012).

Insulin resistance of glucose uptake during pregnancy and lactation

Nowadays it is generally accepted that dairy cows are insulin resistant at the end of gestation and in early lactation. These homeorhetic adaptations are necessary to ensure sufficient glucose supply for the gravid uterus and lactating mammary gland in support of the growing offspring both prenatally and postnatally (Bell and Bauman, 1997). The adaptation towards an insulin resistant state seems to be conserved in mammals among different species. Different studies have been performed to assess insulin resistance of the glucose metabolism during pregnancy and lactation in ruminants and are summarized in Table 5. Overall, most of these studies

confirm a state of increased insulin resistance of the glucose metabolism during early lactation.

The onset of this lactational insulin resistance may be traced to the end of pregnancy.

Table 5: Different studies assessing insulin resistance in ruminants during lactation and pregnancy

Author	Species	Test	Conclusion
(Vernon et al., 1990)	sheep	HEC test with measurement of arteriovenous glucose difference in the hindlimb	Decreased sensitivity and decreased responsiveness of the insulin stimulated glucose uptake in skeletal muscles of lactating animals versus nonlactating and nonpregnant animals
(Vernon and Taylor, 1988)	sheep	in vitro culture adipose tissue	Decreased responsiveness and decreased sensitivity of the insulin stimulated glucose uptake in adipose tissue of lactating sheep versus nonlactating and nonpregnant animals
(Debras et al., 1989)	goat	HEC test	Decreased responsiveness of the whole body insulin stimulated glucose uptake in goats in early lactation versus late lactating and dry goats
(Kerestes et al., 2009)	dairy cow	IVITT	Lower insulin stimulated blood glucose reduction in early lactating dairy cows versus dry and midlactating cows
(Pettersen et al., 1993)	sheep	HEC test	Decreased insulin sensitivity of the whole body insulin stimulated glucose uptake in late pregnant sheep versus nonpregnant and nonlactating sheep
(Ji et al., 2012)	dairy cow	in vitro culture adipose tissue	Reduced insulin signaling (IRS-1 tyrosine phosphorylation) in the adipose tissue of early lactating versus dry dairy cows

Adipose tissue and its derivatives

The adipose tissue and its derivatives undoubtedly play a crucial role in the determination and modulation of insulin sensitivity of the glucose metabolism in dairy cows. A first indication in this direction was given by McCann and Reimers (1985). Using the intravenous insulin tolerance test, they demonstrated a lower reduction of glucose in overconditioned heifers when compared with lean heifers (McCann and Reimers, 1985).

In the last decade, different researchers have focused on the role of NEFA in the insulin sensitivity of dairy cattle. In experimental studies using nonpregnant and nonlactating dairy cows, an artificial elevation of circulating NEFA following a fasting period or by the intravenous administration of a tallow infusion has been shown to cause an impaired insulin stimulated glucose uptake by insulin sensitive tissues (Oikawa and Oetzel, 2006; Pires et al.,

2007b; Schoenberg et al., 2012). On the other hand, reduction of the NEFA load by means of abomasal delivery of nicotinic acid improved the insulin stimulated glucose uptake (Pires et al., 2007a).

Other studies provided evidence for the extrapolation of these experimental results in practice. Overfeeding dairy cows during the dry period provoked an excessive condition score at parturition (Agenas et al., 2003). In the dry period, these overfed cows showed higher peripheral levels of insulin in response to a glucose bolus, which may point to a higher insulin resistance of the peripheral tissues eliciting a secondary hyperinsulinemia at that time. In the subsequent lactation, these overfed cows showed prolonged elevated levels of NEFA and a lower glucose clearance after an intravenous glucose tolerance test, indicating a higher degree of insulin resistance (Holtenius et al., 2003). In dairy cows suffering from different degrees of fatty liver, NEFA levels were negatively correlated with the insulin stimulated reduction in blood glucose after an insulin challenge (Ohtsuka et al., 2001). In a study using cows suffering from different forms of ketonemia, the cows with chronically elevated concentrations of BHB (more than 1 mmol/L from 2 days preceding calving until 7 days following calving) showed a higher insulin resistance of the glucose metabolism at their peripheral tissues. In the same study, a higher concentration of NEFA was significantly correlated with a lower insulin secretion (Kerestes et al., 2009). These findings suggest an important role of NEFA in the development of insulin resistance.

Underlying molecular mechanisms of fatty acid induced insulin resistance are as yet unstudied in dairy cows but may be extrapolated from human medicine. A first mechanism is called the Randle cycle or glucose-fatty acid cycle, which involves the biochemical mechanisms that control substrate (glucose or fatty acids) use in skeletal muscle, heart, liver and pancreas (Hue and Taegtmeyer, 2009). The Randle cycle postulates that when availability of fatty acids is abundant, the oxidation of the latter inhibits the use of glucose as substrate for the cellular metabolism. This process is mediated by different intracellular pathways: inhibition of the pyruvate dehydrogenase, phosphofructokinase activity and hexokinase activity and a decreased GLUT4 translocation in skeletal muscle (Hue and Taegtmeyer, 2009). Additionally, NEFA induce the phosphorylation of insulin receptor substrate 1 (IRS-1) on serine residues. The

serine phosphorylation of IRS1 leads to a decrease in insulin induced tyrosine phosphorylation of IRS1 which is necessary for normal activation of the insulin signaling cascade (LeMarchand-Brustel et al., 2003).

Besides NEFA, other (unknown) factors originating from the adipose tissues also affect the insulin stimulated glucose uptake. As demonstrated in obese sheep, insulin sensitivity is decreased despite their NEFA levels being equally as high as those in lean sheep (Bergman et al., 1989). In human medicine, the metabolic syndrome links visceral obesity, insulin resistance, dyslipidemia and hypertension (Cornier et al., 2008). The adipose tissue plays a central role in the development of the metabolic syndrome by increasing the production of pro-inflammatory adipokines (TNF, IL6, MCP-1) and decreasing the production of the anti-inflammatory adipokine, adiponectin (Gustafson et al., 2007). In dairy cows, the endocrine function of the adipose tissue has been confirmed by demonstrating mRNA expression for TNF, IL6, MCP1, leptin, adiponectin, haptoglobin, visfatin and resistin (Ingvarsen and Boisclair, 2001; Komatsu et al., 2003; Lemor et al., 2009; Mukesh et al., 2010; Sadri et al., 2010; Saremi et al., 2012). The effect of these adipokines on insulin stimulated glucose uptake by the skeletal muscle and adipose tissue is unknown in cattle, with the exception of TNF. Prolonged exposure to TNF, by means of a series of subcutaneous injection, induced insulin resistance in young steers and increased triglyceride concentration in the liver of dairy cows (Kushibiki et al., 2001a; Kushibiki et al., 2001b; Bradford et al., 2009). Ohtsuka et al. concluded that cows suffering from fatty liver had higher serum concentrations of NEFA, were more insulin resistant and had higher serum TNF activity (2001). These observations, together with the study of O'Boyle et al. (2006) in which it was shown that overconditioned cows have higher plasma TNF concentrations, lead the authors to hypothesize that overconditioning in dairy cows may lead to a state of chronic low-grade inflammation, causing insulin resistance, metabolic disorders and rendering these cows to hyperinflammatory reactions in case where infections occur.

Genetic influences on the effect of insulin on the glucose metabolism

As in humans, in cattle there also seems to be a genetic component that determines the sensitivity to insulin resistance. Cows with a high genetic merit for milk production (North

American cows) are stated to be more insulin resistant than cows with a low genetic merit for milk production (New Zealand cows) (Chagas et al., 2009). In an attempt to identify a gene that may influence the effect of insulin on the glucose metabolism, Balogh et al. (2008) performed intravenous glucose tolerance tests in dairy cows 10 - 15 days post partum. Differences in growth hormone Alu1 genotype suggested (non-significantly) that heterozygous (leucine - valine) dairy cows suffered a higher degree of insulin resistance in comparison to homozygous (leucine - leucine) dairy cows (Balogh et al., 2008). These studies suggest that the genetic component modulating insulin resistance might be subtle and may be overwhelmed by other factors contributing to the determination of insulin resistance.

The (epi)genetic determination of insulin sensitivity and insulin resistance has been suggested by Bossaert et al. (2009). By performing IVGTT and IVITT, Bossaert et al. demonstrated that female Holstein Friesian calves (bred for milk production) as early as age 15 days, had higher insulin secretion and lower glucose clearance in comparison with female Belgian Blue calves (bred for beef production), thus suggesting that Holstein Friesian calves are more insulin resistant than Belgian Blue calves (Bossaert et al., 2009).

The apparent 'genetic' differences in insulin resistance for glucose metabolism may be determined by genetic or epigenetic differences between animals. Further investigation is required to define genes that influence insulin resistance and to describe epigenetic effects on these genes.

3.10.5. Release of nonesterified fatty acids from the adipose tissue

NEFA release from the adipose tissue is the result of the combined effect of lipolysis of triglycerides and re-esterification (lipogenesis) of NEFA in the adipocytes (Chilliard et al., 2000). Both pathways are influenced by insulin. In early lactation, blood NEFA levels rise owing to increased lipolysis and decreased lipogenesis partly mediated by reduced serum insulin concentrations during this time (Chilliard et al., 2000). Faulkner and Pollock (1990) and Petterson et al. (1994) demonstrated a state of insulin resistance of the lipid metabolism in pregnant and lactating sheep. Research is limited regarding insulin resistance of lipolytic and lipogenic pathways in the adipose tissue of dairy cows.

Lipolysis and lipogenesis are metabolic pathways influenced by different hormones and changes in these pathways differ between different fat depots (subcutaneous and abdominal) in the same animal. In sheep, omental adipocytes show higher rates of basal and catecholamine stimulated lipolysis in comparison with subcutaneous adipose tissue (Vernon et al., 1995). In dairy cows, subcutaneous adipose tissue shows higher rates of catecholamine stimulated lipolysis and higher rates of lipogenesis in comparison with omental adipose tissue (Smith and Walsh, 1988). This situation might result from a different degree of expression and phosphorylation of hormone sensitive lipase (Locher et al., 2011). On the other hand, Hostens et al. (2012) recently showed that in cows suffering from left displacement of the abomasum, the fatty acid profile of the circulating NEFA was more closely associated to the fatty acid profile of the abdominal fat than the subcutaneous fat (Hostens et al., 2012), suggesting that under these conditions cows preferentially break down the abdominal fat. More research on the metabolic properties and insulin resistance of the different fat depots is needed to quantify the relative contribution of the different fat depots to the NEFA level post partum and to identify the locations of fat deposition that are associated with the greatest negative impact on metabolic adaptation during the transition period.

3.10.6. Glucose output of the liver

The output of glucose from the liver is the result of two metabolic processes: gluconeogenesis and glycogenolysis. Quantitatively, glycogenolysis is of minor importance since glycogen stores in the liver are rather limited and are quickly exhausted in periods of high glucose requirements (Veenhuizen et al., 1991; Herdt, 2000; Karcagi et al., 2008). Gluconeogenesis is by far the most important metabolic pathway in ruminants and has to be tightly regulated. Regulation occurs through both nutritional (substrate supply) and hormonal factors (insulin, glucagon, somatotropin, cortisol) (Danfaer, 1994; Drackley et al., 2001a).

The net effect of insulin on the liver is a decrease in glucose release into the blood, which results from an inhibitory effect of insulin on glycogenolysis and gluconeogenesis (DeFronzo, 2004). The inhibitory effect of insulin on the gluconeogenesis is due to both direct and indirect effects. Insulin directly inhibits key enzymes in the gluconeogenic pathway while indirectly

reducing the availability of glucogenic precursors by its influence on peripheral tissues (stimulation of protein synthesis, inhibition of lipolysis) (Danfaer, 1994).

Insulin decreases gluconeogenesis but increases the proportional use of propionate as substrate for the gluconeogenesis (Brockman, 1990). This process can be explained by the inhibitory effect of insulin on protein, glucose and triglyceride catabolism and the stimulatory effect of insulin on protein, glucose and triglyceride anabolism thereby decreasing the amount of circulating amino acids, lactate and glycerol and thus decreasing their availability for use in gluconeogenesis. The supply of propionate is mainly regulated by feed intake, thus its availability increases relative to the other substrates under influence of insulin.

In humans suffering from type 2 diabetes, the liver is resistant to the inhibitory effect of insulin on hepatic glucose production. This resistance is responsible for the hyperglycemia observed in persons with type 2 diabetes (DeFronzo, 2004). In lactating dairy cows, such an insulin resistant state of the hepatic glucose output would not result in hyperglycemia because of the high glucose uptake by the mammary gland. However, pregnancy and lactation do not alter the insulin response of hepatic glucose production in sheep and goats (Debras et al., 1989; Faulkner and Pollock, 1990; Petterson et al., 1993).

3.10.7. Protein metabolism of skeletal muscle, liver and other peripheral tissues

Insulin influences protein metabolism by stimulation of protein synthesis and inhibition of protein degradation in skeletal muscle and other tissues. Very few studies report on the insulin sensitivity and insulin responsiveness of these metabolic processes. Using the hyperinsulinemic euglycemic euaminoacidemic clamp test in lactating and dry goats, Tesseraud et al. (1993) found that there was no stimulatory effect of insulin on protein synthesis but that inhibition of protein degradation by insulin was enhanced in early lactation. This observation seems to be in conflict with the observations that peripheral tissues (skeletal muscle) decrease amino acid use and increase amino acid mobilization in early lactation (Baracos et al., 1991; Kuhla et al., 2011). However, early lactation is characterized by low insulin levels which leads to a reduced protein synthesis in skin, skeletal muscle and other peripheral tissues (Baracos et al., 1991) and decreased inhibition of protein degradation. The

net result is an increased flow of amino acids to the mammary gland and the liver for synthesis of milk protein and hepatic gluconeogenesis (Kuhla et al., 2011).

3.11. How to measure insulin resistance?

3.11.1. Important considerations

To measure insulin sensitivity in cows, it is important to keep in mind that in dairy cows, the majority of the glucose disappearance occurs independently of insulin, because of the massive glucose drain to the lactating mammary gland and the gravid uterus. In the basal (= non-insulin stimulated) state, the estimated insulin independent glucose uptake is 84% and 92% in dry and lactating cows respectively (Figure 10, calculations based on Overton (1998) and Rose et al. (1997)). The difference between dry and lactating cows is solely due to the higher glucose uptake by the mammary gland in comparison with the gravid uterus (Figure 10).

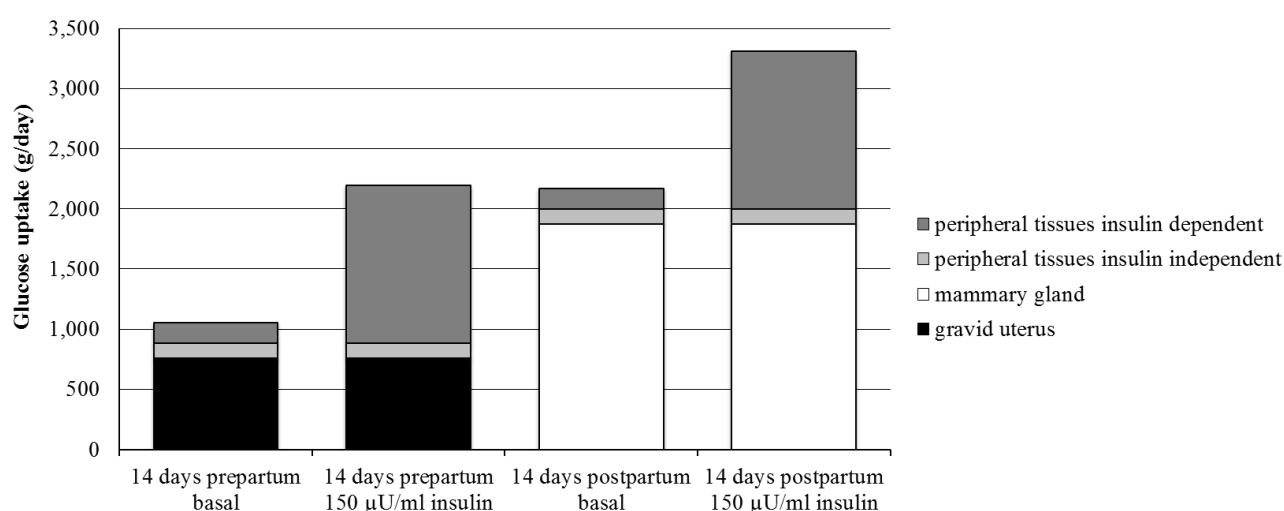


Figure 10. Estimated glucose uptake (g/day) by different tissues in the basal and insulin stimulated (150 µU/ml) state in dairy cows 14 days pre partum and 14 days post partum (26 kg milk) with the same insulin sensitivity (assuming that insulin dependent glucose uptake is equal in prepartum and postpartum cows), based on calculations as described by Overton (1998) and data of Rose et al. (1997).

The higher glucose uptake by the mammary gland may be illustrated by the fact that the basal (non-insulin stimulated) glucose disappearance is 3 times higher in lactating versus dry goats (Debras et al., 1989). Based on the calculated glucose uptake by the different tissues, basal glucose disappearance in lactating dairy cows is 2 times higher compared with the dry period (Figure 10: 14 days pre- and postpartum basal). This difference may become larger depending on the level of milk production (3 to 4 times higher basal glucose disappearance when milk production achieves 40 and 50 kg of milk, respectively). In Figure 10, the estimated glucose

uptake is depicted if one stimulates glucose uptake by an intravenous insulin infusion reaching a serum insulin steady state concentration of 150 $\mu\text{IU/ml}$, assuming (not necessarily true) that there is no difference in insulin sensitivity in dry versus lactating cows (Figure 10: in the insulin stimulated state, the insulin dependent glucose uptake by the peripheral tissues is of the same order in lactating and dry cows). By infusion of insulin, hepatic and renal gluconeogenesis are inhibited, thus glucose infusion has to take over the body's total glucose provision. This process would lead to a higher infusion rate in lactating cows (Figure 10: 3300 g/day vs. 2200 g/day in dry cows) because basal glucose turnover is higher. To compare insulin sensitivity between dry and lactating cows or lactating cows with different milk yield, it is important to consider this difference in basal glucose disappearance otherwise insulin sensitivity of lactating cows may be overestimated. By infusion of stable or radioactive isotopes of glucose in the basal state, it is possible to estimate and correct for this difference in basal glucose disappearance (Debras et al., 1989; Rose et al., 1997).

3.11.2. Hyperinsulinemic euglycemic clamp test

The hyperinsulinemic euglycemic clamp (HEC) test as described by DeFronzo et al. is considered the gold standard method to measure insulin resistance in humans and animals (DeFronzo et al., 1979). A constant insulin infusion raises serum insulin levels to a steady state level after 60 to 90 minutes of infusion (hyperinsulinemic state). During the insulin infusion, blood samples are taken at regular intervals (5 to 10 minutes) to measure blood glucose. Based on the blood glucose measured, the speed of a simultaneous glucose infusion is empirically adapted to clamp blood glucose at the normal basal value (euglycemic). After some time (last hour or last half hour of a 2 hour test) a steady state is reached whereby the serum insulin concentration is constant (steady state insulin concentration = SSIC) and no or minor changes of the glucose infusion (steady state glucose infusion rate = SSGIR) are needed to keep the blood glucose constant at the basal level (DeFronzo et al., 1979; Muniyappa et al., 2008).

The speed of the SSGIR is directly related to the insulin response of the glucose metabolism of the peripheral tissues: a high SSGIR means high glucose uptake per unit insulin (low insulin resistance) whereas a low SSGIR means low glucose uptake per unit insulin (high insulin resistance). This tenet holds true under the assumption that serum insulin concentrations are

high enough to inhibit endogenous glucose production. In ruminants, maximal reduction of hepatic gluconeogenesis is achieved at insulin concentrations of 100-120 $\mu\text{IU}/\text{ml}$ (Brockman and Laarveld, 1986; Petterson et al., 1993). The use of isotopes of glucose allows one to measure the insulin sensitivity of the endogenous glucose production (Muniyappa et al., 2008). The assessment of the insulin response may be based on a single insulin infusion or complete dose response curves may be generated by using different insulin infusions consecutively (Muniyappa et al., 2008). Maximal insulin stimulated whole body glucose disposal in dairy cows is achieved with insulin infusions of 5-10 $\text{mIU}/\text{kg}/\text{min}$ and serum insulin concentrations of 500-1000 $\mu\text{IU}/\text{mL}$ (Kräft, 2004; Kusenda, 2010; Haarstrich, 2011). Using data from HEC tests, one may assess insulin sensitivity by calculating an insulin sensitivity index (ISI). Different calculations have been described:

- $\text{ISI} = \text{SSGIR} / \text{SSIC}$ (Mitrakou et al., 1992);
- $\text{ISI} = \text{SSGIR} / (\text{steady state glucose concentration} * \Delta\text{I})$ (Muniyappa et al., 2008);

where ΔI is the difference between SSIC and basal insulin concentration.

Measurements of NEFA during the HEC test may give an idea of the effect of insulin on lipolysis and lipogenesis and hence may provide an indication of the degree of insulin resistance in adipose tissue (Kräft, 2004).

Major disadvantages of the HEC test are that it is time consuming (several hours for every insulin infusion), very intensive, expensive and requires some experience to constantly adapt the GIR to clamp the blood glucose at the basal level. Another drawback is that the insulin response is measured at supraphysiological insulin levels, limiting the extrapolation to physiological insulin concentrations. Furthermore, the HEC test is not suitable for performance under field conditions or for use in studies with a large number of animals (Muniyappa et al., 2008).

3.11.3. Intravenous glucose tolerance test

The intravenous glucose tolerance test (IVGTT) is a more practical method to assess insulin resistance in dairy cows. After the intravenous infusion of a glucose bolus, blood samples are taken at regular intervals to determine glucose and insulin concentrations. The amount of glucose infused differs between studies from 150 mg/kg (e.g. Holtenius et al., 2003), 250 mg/kg

(e.g. Pires et al., 2007b) to 500 mg/kg (e.g. Kerestes et al., 2009) as does the time frame of sampling: during 180 minutes (e.g. Pires et al., 2007b) or during 60 minutes (e.g. Bossaert et al., 2008c). The IVGTT is easy to perform in practice, although the interpretation of the obtained data requires some insight into glucose and insulin metabolism.

Blood glucose levels during an IVGTT are the resultant of the glucose bolus, endogenous glucose production in liver and kidney, glucose uptake by the intestines, renal glucose excretion, glucose uptake by the mammary gland and/or the gravid uterus and glucose uptake by the insulin sensitive tissues (skeletal muscle and adipose tissue) (Pires et al., 2007b; Bossaert et al., 2009). Blood insulin levels during an IVGTT are the resultant of the insulin secretion by the pancreas in response to the glucose bolus and the elimination of insulin by the liver. The combination of these physiological processes results in the typical profile of insulin and glucose concentrations during an IVGTT.

Based on the data obtained during an IVGTT, calculations may be done to identify differences between animals. Following parameters may be calculated (Pires et al., 2007b; Bossaert et al., 2009):

- clearance rate of glucose and insulin;
- area under the curve for both glucose and insulin;
- time to reach half of the maximal glucose and insulin concentration;
- time to reach the basal glucose and insulin concentration.

Insulin resistance can be assumed when glucose clearance is low, the area under the curve for glucose is high, the time to reach half of the maximal glucose concentration and the time to reach basal glucose concentration are high. Exact interpretation of the test implies normal insulin secretion from the pancreas following administration of the glucose bolus and assumes similar insulin secretion between animals. However, the latter is not always the case. Therefore, a modified IVGTT was developed whereby an additional insulin or tolbutamide bolus is given 20 minutes after the administration of the glucose bolus to evoke an artificial elevation of the blood insulin levels which increases the comparability between subjects (Muniyappa et al., 2008).

Besides the aforementioned calculations, data from the IVGTT may be entered in a mathematical model, ‘the Minimal Model’ as developed by Bergman et al. (1979). The major advantage of this model is that it simultaneously models insulin and glucose concentrations and uses both models to assess the insulin sensitivity (Muniyappa et al., 2008). A computer program, MINMOD, has been developed to facilitate calculations (Boston et al., 2003). Using this program, the following parameters may be calculated (Boston et al., 2003; Cnop et al., 2007; Muniyappa et al., 2008):

- Si or insulin sensitivity index: fractional glucose disappearance per unit insulin;
- Sg or glucose effectiveness at zero insulin: the ability of glucose to stimulate its own disappearance and inhibit endogenous glucose production;
- AIRg or the acute insulin response to glucose: the increase of insulin after the glucose bolus;
- β -cell function: insulin secretory capacity of the pancreatic β -cells;
- DI or disposition index: a measure of β -cell function in relation to the insulin sensitivity.

The IVGTT has been widely used in dairy cows, however it has only recently been compared with the HEC test in dry cows on a different feeding level before and after fasting. Results indicated good agreement between these methods (Schoenberg et al., 2012). The applicability of the Minimal Model in dairy cows requires some additional investigation.

3.11.4. Intravenous insulin tolerance test

The intravenous insulin tolerance test (IVITT) consists of an intravenous bolus injection of insulin and subsequent blood sampling at regular intervals for the measurement of blood glucose. In dairy cows, insulin doses of 0.02 IU/kg (e.g. McCann and Reimers, 1985), 0.05 IU/kg (e.g. Oikawa and Oetzel, 2006) and 0.1 IU/kg (e.g. Pires et al., 2007b) have been used. The following parameters may be calculated using data obtained from IVITT (Pires et al., 2007b; Bossaert et al., 2009):

- glucose clearance rate following the administration of the insulin bolus;
- insulin stimulated reduction in blood glucose (% of basal glucose);
- area under the curve for glucose following the administration of the insulin bolus.

The larger the insulin stimulated reduction in blood glucose and glucose clearance, the higher the insulin response of the glucose metabolism of the peripheral tissues.

Major disadvantages of the IVITT include the elicited hypoglycemia which may generate counterregulatory mechanisms that might confound the estimation of insulin sensitivity. Furthermore, potentially dangerous neurological and cardiovascular side effects of the hypoglycemia may arise if an overdose of insulin is given (Ferrannini and Mari, 1998; Muniyappa et al., 2008).

3.11.5. *Surrogate indices for insulin resistance*

Several surrogate indices to estimate the level of insulin resistance have been developed in human medicine and have subsequently been copied for use in veterinary medicine. The major purpose of their use is to predict insulin resistance of the peripheral tissues based on a single blood sample after an overnight fast (Muniyappa et al., 2008). The following surrogate indices have been proposed (Katz et al., 2000; Perseghin et al., 2001; Rabasa-Lhoret et al., 2003; Balogh et al., 2008; Muniyappa et al., 2008):

$$\text{HOMA - IR} = \text{glucose (mM)} \times \text{insulin } (\mu\text{IU/mL});$$

$$\text{QUICKI} = \frac{1}{\log(\text{glucose (mg/dL)}) + \log(\text{insulin } (\mu\text{IU/mL}))};$$

$$\text{RQUICKI} = \frac{1}{\log(\text{glucose (mg/dL)}) + \log(\text{insulin } (\mu\text{IU/mL})) + \log(\text{NEFA (mM)})};$$

$$\text{RQUICKI}_{\text{BHB}} = \frac{1}{\log(\text{glucose (mg/dL)}) + \log(\text{insulin } (\mu\text{IU/mL})) + \log(\text{NEFA (mM)}) + \log(\text{BHB (mM)})};$$

$$\text{RQUICKI}_{\text{glycerol}} = \frac{1}{\log(\text{glucose (mg/dL)}) + \log(\text{insulin } (\mu\text{IU/mL})) + \log(\text{glycerol}(\mu\text{M}))}.$$

Homeostasis model assessment or HOMA is a mathematical model that predicts the fasting glucose and insulin concentration of an individual using the best possible combination of insulin resistance and insulin secretion. The mathematical model can be simplified to the formula of HOMA-IR or homeostasis model of insulin resistance using the formula shown (Muniyappa et al., 2008). The lower the HOMA-IR value, the lower the insulin resistance of an individual.

The QUICKI or quantitative insulin sensitivity check indices are mathematical calculations based on the logarithmic transformation of the fasting glucose and insulin concentrations

including some other blood parameters (NEFA, BHB, glycerol). These indices have been proven to be very useful in human medicine (Muniyappa et al., 2008). QUICKI indices may be interpreted such that lower values are indicative of higher insulin resistance of an individual. Some of the surrogate indices have been adopted in veterinary medicine and have been used to predict insulin resistance in dairy cows. However, the applicability of these indices requires further investigation. Indeed, the fasting state in humans is crucial if one is to obtain reliable information on insulin resistance derived from these surrogate indices (Muniyappa et al., 2008). In dairy cows, with the exception of suckling calves, it is impossible to achieve a fasting state whereby insulin and glucose levels are in a balanced state. In addition, the glucose, insulin, NEFA, BHB and glycerol levels of dairy cows can change tremendously at the end of pregnancy and during early lactation, thereby reducing the suitability of these indices to be applied during these periods. Any kind of stress situation during sampling can furthermore lead to important changes in glucose, NEFA and insulin which will affect the interpretation of the indices (Leroy et al., 2011). Besides these *in vivo* considerations regarding the application of surrogate indices, laboratory techniques may also affect the application of surrogate indices. Analysis of insulin concentrations in bovine serum requires the application of analytical procedures specifically developed for bovine insulin. Since cross reactivity with human insulin is not 100%, it is advised to use bovine specific kits to calculate the surrogate indices for insulin sensitivity and thus allowing comparison between different studies (Abuelo et al., 2012).

Nevertheless, some of these indices have been applied in different studies dealing with dairy cows (Holtenius and Holtenius, 2007; Balogh et al., 2008; Kerestes et al., 2009; Schoenberg et al., 2011; Schoenberg et al., 2012). Some studies reported similar changes in values for QUICKI, RQUICKI and RQUICKI_{BHB} and parameters of GTT (Balogh et al., 2008; Bossaert et al., 2009; Kusenda, 2010; Haarstrich, 2011), others however failed to support these observations (Kerestes et al., 2009). The practical application of these indices requires further validation in dairy cows.

3.11.6. Other tests

More invasive tests may be used in order to assess the tissue specific insulin response in skeletal muscle and adipose tissue. Vernon et al. determined the arteriovenous glucose difference in the hind limb of lactating and nonlactating sheep using arterial and venous catheters during a hyperinsulinemic euglycemic clamp test to assess the insulin response of the hindlimb muscles (Vernon et al., 1990).

Taking muscle biopsies during a HEC test gives the opportunity to study the activation of the intracellular insulin signaling cascade (Kruszynska et al., 2002). This approach opens the possibility to identify the signaling molecule that is responsible for inducing insulin resistance. Besides in vivo testing, in vitro research of biopsies may provide information concerning the insulin response of specific metabolic pathways.

Short term in vitro cultures of adipose tissue samples may render information about the insulin response of the lipolytic and lipogenic pathways in adipose tissue biopsies and may provide dose response curves to assess differences in insulin sensitivity and insulin responsiveness (Green and Newsholme, 1979; Arner, 1997).

Performing such studies in dairy cows would provide insight into the insulin response of the different metabolic pathways in the different insulin sensitive tissues and may help to identify the underlying pathogenesis of insulin resistance at the molecular level. However, these studies are time consuming, require experience of in vivo and in vitro techniques and cannot be performed on large scale.

4. CONCLUSIONS

The recent developments in research on the pathophysiology of the metabolic syndrome in human medicine have revealed an important endocrine function of the adipose tissue. The adipose tissue is no longer considered to be only a storage place for excess energy; it is also capable of secreting a wide array of bioactive molecules, named adipokines. These adipokines seem to be the missing link between obesity, cardiovascular disease and insulin resistance in human medicine.

In dairy cows, in which overconditioning at calving means an increased risk for reproductive, infectious and metabolic disorders in the transition period, we can no longer ignore the

secretory capacity of the adipose tissue. This new perspective on the role of the adipose tissue in these disorders highlights the importance of monitoring the BCS during the lactation cycle and might give new opportunities for the treatment and the management of transition dairy cows.

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CHAPTER 3

Aims

The general aim of the present doctoral thesis was to identify key factors in the pathogenesis of the increased disease susceptibility in overconditioned dairy cows during the transition period. More specifically, we aimed to assess the role of the accumulation of adipose tissue on the development of insulin resistance in dairy cows at the end of the dry period.

Measuring insulin resistance in dairy cows is complicated by the fact that dairy cows are lactating and/or pregnant. These are two physiological states which utilize glucose independently of insulin making it difficult to assess the insulin dependent glucose uptake. In Chapter 4 we aimed to get a better insight in different ways to assess insulin sensitivity in dairy cows by performing different studies:

1. In a first study, we aimed to compare different measures of insulin sensitivity with the gold standard method, being the hyperinsulinemic euglycemic clamp test, in pregnant dairy cows at the end of pregnancy to verify which measures of insulin sensitivity can be used reliably in dairy cows at the end of pregnancy (Chapter 4.1).
2. In a second study, we aimed to identify the influence of different physiological states (pregnancy and lactation) on measures of insulin sensitivity derived from a routinely used test, being the glucose tolerance test (Chapter 4.2).

By performing hyperinsulinemic euglycemic clamp tests in dairy cows with a variable body condition score, we aimed to assess the relationship between overconditioning in dairy cows and peripheral tissue insulin resistance. Previous research demonstrated that the glucose and fatty acid metabolism might react differently on insulin. Therefore, we aimed to assess insulin sensitivity and insulin responsiveness of the glucose and fatty acid metabolism separately (Chapter 5).

Because excessive mobilization of body fat is an important risk factor for the development of periparturient disorders, the aim of Chapter 6 was to determine factors influencing basal and stimulated lipolytic activity as well as insulin sensitivity of the lipolytic activity in subcutaneous and omental adipocytes. More specifically, we were interested in assessing whether adipocyte size and adipose depot influenced lipolytic properties of the adipocytes.

Obesity in humans is associated with a chronic pro-inflammatory state originating from a dysregulated production of adipokines by the adipose tissue. Therefore, in Chapter 7, we aimed

to assess if a similar dysregulated production of adipokines plays a role in the pathogenesis of the increased disease susceptibility in overconditioned dairy cows during the transition period:

1. In a first study, we aimed to assess the expression of different adipokines in different adipose depots to identify the impact of excessive accumulation of body fat on the expression of adiponectin, leptin, IL6, TNF and CD14 in dairy cows at the end of the dry period (Chapter 7.1).
2. In a final study, we aimed to assess the circulating concentration of adiponectin and its association with the peripheral tissue insulin sensitivity of cows during the dry period (Chapter 7.2).

CHAPTER 4

Measuring insulin sensitivity

CHAPTER 4.1

Validation of different measures of insulin sensitivity of glucose metabolism in dairy cows using the hyperinsulinemic euglycemic clamp test as the gold standard

De Koster J., Hostens M., Hermans K., Van den Broeck W. and Opsomer G. 2016. Validation of different measures of insulin sensitivity of glucose metabolism in dairy cows using the hyperinsulinemic euglycemic clamp test as the gold standard. Domestic Animal Endocrinology. 57: 117-126.

1. ABSTRACT

The aim of the present research was to compare different measures of insulin sensitivity in dairy cows at the end of the dry period. To do so, ten clinically healthy dairy cows with a varying body condition score were selected. By performing hyperinsulinemic euglycemic clamp (HEC) tests, we previously demonstrated a negative association between the insulin sensitivity and insulin responsiveness of glucose metabolism and the body condition score of these animals. In the same animals, other measures of insulin sensitivity were determined and the correlation with the HEC test, which is considered as the gold standard, was calculated. Measures derived from the intravenous glucose tolerance test (IVGTT) are based on the disappearance of glucose after an intravenous glucose bolus. Glucose concentrations during the IVGTT were used to calculate the area under the curve of glucose and the clearance rate of glucose. Additionally, glucose and insulin data from the IVGTT were fitted in the minimal model to derive the insulin sensitivity parameter, S_i . Based on blood samples taken before the start of the IVGTT, basal concentrations of glucose, insulin, NEFA and β -hydroxybutyrate (BHB) were determined and used to calculate surrogate indices for insulin sensitivity, such as the homeostasis model of insulin resistance (HOMA-IR), the quantitative insulin sensitivity check index (QUICKI), the revised quantitative insulin sensitivity check index (RQUICKI) and the revised quantitative insulin sensitivity check index including BHB ($RQUICKI_{BHB}$). Correlation analysis revealed no association between the results obtained by the HEC test and any of the surrogate indices for insulin sensitivity. For the measures derived from the IVGTT, the area under the curve of glucose for the first 60 min of the test and the S_i derived from the minimal model demonstrated good correlation with the gold standard.

2. INTRODUCTION

Insulin resistance is defined as a state where a normal concentration of insulin evokes a less than normal biological reaction (Kahn, 1978). Development of a transient state of insulin resistance at the end of pregnancy and the beginning of lactation is an important homeorhetic adaptation mechanism of mammals to preserve sufficient glucose for the growing fetus and the nursing neonate (Bell and Bauman, 1997; De Koster and Opsomer, 2013). In dairy cows genetically selected for high milk production, these homeorhetic adaptation mechanisms are

driven to extremes (Chagas et al., 2009). Insulin resistance in the transition period has been associated with several pathological conditions like ketosis and cystic ovarian disease (Opsomer et al., 1999; Kerestes et al., 2009). Several researchers have tried to identify risk factors for the development of increased insulin resistance (Pires et al., 2007; De Koster et al., 2015) or investigated potential modifying effects of nutritional strategies (Holtenius et al., 2003; Schoenberg et al., 2011; Mann et al., 2016) or nutritional (Hayirli et al., 2001; Pires et al., 2008) or pharmacological (Smith et al., 2007; Schoenberg et al., 2011; Kusenda et al., 2013) substances on the degree of peripheral tissue insulin sensitivity in dairy cows. The conclusions are difficult to appraise and compare because these investigations used different and often non-validated methods to assess insulin sensitivity in dairy cows.

The gold standard to measure insulin sensitivity is the hyperinsulinemic euglycemic clamp (HEC) test described by DeFronzo et al. (1979). Under hyperinsulinemic conditions, the concomitantly infused glucose is taken up primarily by insulin sensitive tissues allowing evaluation of peripheral tissue insulin sensitivity and responsiveness. Unfortunately, HEC tests are laborious and expensive, therefore other tests to evaluate insulin sensitivity have been developed. The disappearance of glucose after an intravenous glucose challenge has frequently been used as a more practical way of measuring insulin sensitivity. The area under the curve (AUC) and the clearance rate (CR) are calculated based on the glucose concentration during the intravenous glucose tolerance test (IVGTT) (Holtenius et al., 2003; Pires et al., 2007). These measures rely on the assumption that the disappearance of glucose will be slower in insulin resistant individuals. Bergman et al. (1979) described the use of a mathematical model, the minimal model, based on the glucose and insulin dynamics during an IVGTT. Based on the parameters derived from this model, an index of insulin sensitivity (S_i) can be calculated. In humans, surrogate indices for insulin sensitivity have been proposed based on the analysis of glucose, insulin, NEFA and β -hydroxybutyrate (BHB) in a single blood sample after an overnight fast. The surrogate indices most frequently used are the homeostasis model of insulin resistance (HOMA-IR), the quantitative insulin sensitivity check index (QUICKI) and the revised quantitative insulin sensitivity check index (RQUICKI) (Perseghin et al., 2001; Muniyappa et al., 2008). These indices have been applied as a measure of insulin sensitivity in

dairy cows as well (Holtenius and Holtenius, 2007; Balogh et al., 2008), but their use is, to the best of our knowledge not yet fully validated and hence questionable (Kerestes et al., 2009; Schoenberg et al., 2012; Mann et al., 2016).

Until now, none of the aforementioned methods to measure insulin sensitivity have been compared with the gold standard method in dairy cows. The aim of the present study was to compare insulin sensitivity in dairy cows at the end of the dry period as measured by the HEC test, the IVGTT or the calculated surrogate indices for insulin sensitivity.

3. MATERIALS AND METHODS

All experimental procedures were approved by the ethical committee of the Faculty of Veterinary Medicine (EC2010/149 - University Ghent, Belgium).

3.1. Study design

Ten clinically healthy, pregnant Holstein Friesian dairy cows (upcoming parity two to five) were selected at the beginning of the dry period based on body condition score (BCS) according to the scale of Edmonson et al. (1989). Five animals were considered to have a normal BCS (BCS 2.5 to 3.5) and 5 animals were considered to be overconditioned (BCS 3.75 to 5). The study design is described in detail by De Koster et al. (2015). Briefly, cows were followed starting 2 months before the expected parturition date. In the third week (21 to 17 d) before the expected parturition date, cows were weighed and catheters (Cavafix Certo 338 - 14G, B. Braun, Instrulife, Oostkamp, Belgium) were placed in both jugular veins. After a resting period of 2 h, an IVGTT was performed. The next day, the animals underwent a HEC test. All infusions were administered through the left jugular catheter while blood samples were taken from the right jugular catheter.

3.2. Surrogate indices for insulin sensitivity

The surrogate indices for insulin sensitivity were calculated using the glucose, insulin, NEFA and BHB concentration as determined in serum samples taken 15 min before the start of the IVGTT. Calculations were performed as described by De Koster and Opsomer (2013):

$$\text{HOMA} - \text{IR} = \text{glucose (mM)} \times \text{insulin} \left(\frac{\mu\text{IU}}{\text{mL}} \right);$$

$$\text{QUICKI} = \frac{1}{\log \left(\text{glucose} \left(\frac{\text{mg}}{\text{dL}} \right) \right) + \log \left(\text{insulin} \left(\frac{\mu\text{IU}}{\text{mL}} \right) \right)};$$

$$\text{RQUICKI} = \frac{1}{\log(\text{glucose (mg/dL)}) + \log(\text{insulin } (\mu\text{IU/mL})) + \log(\text{NEFA (mM)})};$$

$$\text{RQUICKI}_{\text{BHB}} = \frac{1}{\log(\text{glucose (mg/dL)}) + \log(\text{insulin } (\mu\text{IU/mL})) + \log(\text{NEFA (mM)}) + \log(\text{BHB (mM)})}.$$

3.3. Intravenous glucose tolerance test

From 2 h before the IVGTT until the end, cows had access to fresh drinking water but not feed. Glucose was infused at a dose of 250 mg/kg body weight (Glucose 30%, Eurovet, Verdifarm, Beringen-Paal, Belgium) over a period of 5 to 10 min, after which the catheter was flushed three times with 20 mL of saline. Time point 0 was the moment when all the glucose was infused. Blood samples for the determination of glucose and insulin were taken at following time points: -15, -5, 0, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 150 and 180 min.

Based on the analyses of the glucose and insulin concentrations during the IVGTT, different measures of insulin sensitivity were calculated. The clearance rate of glucose between 0 and 30 min (CR_{0-30}) and 0 and 60 min (CR_{0-60}) were calculated as described by Pires et al. (2007). The area under the curve of glucose was calculated between 0 and 60 min (AUC_{0-60}), between 0 and 120 min (AUC_{0-120}) and between 0 and 180 min (AUC_{0-180}) as the incremental AUC using the trapezoidal rule as described by Cardoso et al. (2011).

3.4. MINMOD analysis of the intravenous glucose tolerance test

Glucose and insulin data derived from the IVGTT were fitted using the MINMOD Millenium program (Boston et al., 2003) based on the minimal model as described by Bergman et al. (Bergman et al., 1979). The derived variable of interest was S_i , a measure of the ability of insulin to enhance the fractional glucose disappearance. For model fitting purposes in the MINMOD program, the results of the IVGTT were adapted as follows: basal glucose concentrations measured at time -15 and -5 min were averaged and assigned to time point 0 min in the MINMOD program; glucose concentrations measured at time 0 min were assigned to time point 2 min in the MINMOD program; results from the first 10 min during the IVGTT were zero-weighted to allow for glucose mixing and basal values for glucose and insulin were added at time points 240 and 300 min (Bergman et al., 1979; Pacini and Bergman, 1986; Boston et al., 2003). The curves generated by the MINMOD program were evaluated by visual

assessment of the fit of the curves with the original data, the fractional standard deviation (< 17%) and the coefficient of determination ($r^2 > 97\%$) (Pacini and Bergman, 1986).

3.5. Hyperinsulinemic euglycemic clamp test

Hyperinsulinemic euglycemic clamp tests were performed as described by De Koster et al. (2015). Briefly, after assessment of the basal blood glucose concentration, 4 consecutive insulin infusions were administered for a period of 2 h each, at increasing doses of insulin: 0.1, 0.5, 2 and 5 mIU/kg per min (Actrapid 100 IU/mL, human recombinant insulin, Novo Nordisk, Bagsvaerd, Denmark). At regular time points (every 2.5 or 5 min), blood glucose concentration was determined using a hand-held glucometer (Precision Xceed, Abbott Diabetes Care, Verdifarm, Beringen-Paal, Belgium) and compared with the basal blood glucose concentration measured before the start of the HEC test. When the blood glucose concentration dropped, the speed of the glucose infusion (Glucose 30%, Eurovet, Verdifarm, Beringen-Paal, Belgium) was increased to keep the blood glucose concentration near basal levels. A steady-state was reached when no or minor changes of the glucose infusion were necessary to keep the blood glucose concentration constant and near basal levels for at least 30 min. During the steady state period, the steady state glucose infusion rate (SSGIR) and the steady state insulin concentration (SSIC) were calculated as the average GIR and the average insulin concentration.

Insulin sensitivity indices were calculated for each insulin infusion period ($ISI_{inf 1}$, $ISI_{inf 2}$, $ISI_{inf 3}$, $ISI_{inf 4}$) using the following formula (Muniyappa et al., 2008; De Koster and Opsomer, 2013):

$$ISI = \frac{SSGIR}{\Delta I \times SSG} ;$$

with ΔI being the difference between the SSIC and the basal insulin concentration and SSG being the steady state glucose concentration. During the HEC test, water and hay were always available but corn silage was withheld from 12 h before and until the end of the test.

3.6. Blood analyses

Samples for glucose determination were taken in fluoride blood tubes (Vacutest, Novolab, Geraardsbergen, Belgium). Samples for NEFA, BHB and insulin determination were taken in gel-coated blood tubes (Vacutest, Novolab, Geraardsbergen, Belgium). Within 2 h after collection, all blood samples were centrifuged for 20 min ($2,460 \times g$, 7°C) and serum and plasma were stored at -80°C until analysis. Glucose concentrations were determined using an

enzymatic reference method, intra- and inter-assay CV were 0.82% and 1.1%, respectively. Serum NEFA concentrations were determined using an enzymatic endpoint method, intra- and inter-assay CV were 1.0% and 1.1%, respectively. Serum BHB concentrations were determined using an enzymatic endpoint method, intra- and inter-assay CV were 0.95% and 2.9%, respectively. Serum insulin concentrations were determined using a commercial ELISA kit (Bovine Insulin ELISA, Catalog nr 10-1201-01, Mercodia, Uppsala, Sweden). Conversion of insulin concentration from gravimetric units to international units was done as described by Abuelo et al. (2012). Intra- and inter-assay CV were 4.7% and 9.5%, respectively. Samples of the HEC test were analyzed as described by De Koster et al. (2015).

3.7. Statistical analyses

All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). One cow was excluded from final statistical analysis because steady state conditions were not reached in 2 of the 4 insulin infusion periods of the HEC test. Descriptive statistics (PROC MEANS) are expressed as mean \pm SEM unless otherwise indicated. Normality of the data (PROC UNIVARIATE) was checked using the Kolmogorov-Smirnov test ($P < 0.05$). Basal insulin concentration and HOMA-IR were not normally distributed and log transformed for further statistical analysis.

Insulin sensitivity indices (ISI) derived from the HEC test were compared with measures derived from the IVGTT (AUC, CR) and surrogate indices for insulin sensitivity (HOMA-IR, QUICKI, RQUICKI, RQUICKI_{BHB}) by calculating Pearson correlation coefficients while relationships were visualized in dot plots. Insulin sensitivity indices (ISI) derived from the HEC test and Si derived from MINMOD analysis of the IVGTT were compared after conversion of values to identical units (dL/min per μ IU/mL) as described by Bergman et al. (1987):

for ISI derived from the HEC test: $ISI_{converted} = ISI \times bodyweight$;

for Si derived from the MINMOD: $Si_{converted} = Si \times \frac{glucose\ dose}{G0 - Gb}$;

with glucose dose being the total administered dose of glucose in mg, G0 being the postinjection glucose concentration and Gb being the basal glucose concentration derived from the MINMOD. Converted values were compared by calculating concordance correlation coefficients (CCC) using the U statistic (Carrasco et al., 2013) and relationships were visualized

in Bland-Altman plots. Significance and tendency were declared at $P < 0.05$ and $0.05 < P < 0.1$, respectively.

4. RESULTS

Data derived from the HEC tests and calculated ISI are given in Table 1. The consecutive insulin infusions increased SSIC incrementally and as a consequence, SSGIR increased concomitantly to keep blood glucose concentration near basal levels. The mean and SEM of the glucose, insulin, NEFA and BHB concentrations used to calculate the surrogate indices are given in Table 2.

Table 1. Steady state insulin concentration (SSIC), steady state glucose infusion rate (SSGIR) and insulin sensitivity indices (ISI) during the different insulin infusions^a of the hyperinsulinemic euglycemic clamp (HEC) test (mean \pm SEM).

Insulin infusion	SSIC (μ IU/mL)	SSGIR (μ mol/kg/min)	ISI ^b ($\times 10^{-2}$ mL/kg/min per μ IU/mL)
Basal	1.09 \pm 0.08	0	0
0.1 mIU/kg per minute	8.52 \pm 0.33	1.44 \pm 0.05	7.09 \pm 1.11
0.5 mIU/kg per minute	51.02 \pm 1.73	8.77 \pm 0.28	5.52 \pm 0.90
2 mIU/kg per minute	337.24 \pm 12.70	13.61 \pm 0.32	1.35 \pm 0.17
5 mIU/kg per minute	1,432.86 \pm 52.46	18.33 \pm 0.45	0.425 \pm 0.069

^a During the HEC test, 4 consecutive insulin infusions were administered at increasing doses of insulin: 0.1, 0.5, 2 and 5 mIU/kg per minute for infusion 1, 2, 3 and 4, respectively.

^b Insulin sensitivity indices were calculated using the following formula $ISI = SSGIR / (\Delta I \times SSG)$ with SSGIR = steady state glucose infusion rate, ΔI = difference between the steady state insulin concentration and the basal insulin concentration and, SSG = steady state glucose concentration during each insulin infusion period.

Table 2. Basal glucose, insulin, NEFA and β -hydroxybutyrate (BHB) concentration^a.

Measure	Mean \pm SEM
Glucose (mg/dL)	67 \pm 1.9
Insulin (μ IU/mL)	9.98 \pm 1.891
NEFA (mM)	0.47 \pm 0.082
BHB (mM)	0.60 \pm 0.043

^a Basal values as measured in serum or plasma taken 15 min before the start of the intravenous glucose tolerance test.

There was no correlation between the BCS and the basal insulin concentration ($r = -0.009$; $P = 0.98$). Very little variation existed in the calculated surrogate indices (Table 3). Pearson correlation coefficients between the different ISI and any of the surrogate indices were weak and not significant (Table 4, Figure 1).

Table 3. Calculated surrogate indices for insulin sensitivity.

Measure ^a	Mean \pm SEM
QUICKI	0.362 \pm 0.009
RQUICKI	0.420 \pm 0.015
RQUICKI _{BHB}	0.466 \pm 0.019
HOMA-IR	37.5 \pm 7.7

^a QUICKI = quantitative insulin sensitivity check index;

RQUICKI = revised quantitative insulin sensitivity check index;

RQUICKI_{BHB} = revised quantitative insulin sensitivity check index including BHB;

HOMA-IR = homeostasis model of insulin resistance.

Table 4. Pearson correlation coefficients between insulin sensitivity indices (ISI) derived from the hyperinsulinemic euglycemic clamp (HEC) test^a and intravenous glucose tolerance test (IVGTT)^b and surrogate indices for insulin sensitivity^c

	ISI _{inf 1}	ISI _{inf 2}	ISI _{inf 3}	ISI _{inf 4}
AUC ₀₋₆₀	-0.53	-0.69*	-0.63 [†]	-0.49
AUC ₀₋₁₂₀	-0.49	-0.64 [†]	-0.53	-0.37
AUC ₀₋₁₈₀	-0.49	-0.59 [†]	-0.48	-0.32
CR ₀₋₃₀	0.35	0.39	0.15	-0.11
CR ₀₋₆₀	0.13	0.29	-0.05	-0.24
QUICKI	0.02	0.17	-0.01	-0.42
RQUICKI	0.03	0.49	0.14	-0.20
RQUICKI _{BHB}	-0.21	0.24	0.02	-0.24
HOMA-IR ^d	-0.05	-0.20	-0.002	0.42

* $P < 0.05$, [†] $0.05 < P < 0.1$

^a Insulin sensitivity indices (ISI) were calculated using the following formula $ISI = SSGIR/(\Delta I \times SSG)$ [mL/kg/min per μ IU/mL], with SSGIR = steady state glucose infusion rate, ΔI = difference between the steady state insulin concentration and the basal insulin concentration and, SSG = steady state glucose concentration during each insulin infusion period.

^b Si = insulin sensitivity derived from the minimal model [$\times 10^{-4}$ (μ IU/mL)⁻¹ per min]; AUC₀₋₆₀ = area under the curve (AUC) of glucose during the first 60 min of the IVGTT [mg/dL \times 60 min]; AUC₀₋₁₂₀ = AUC of glucose during the first 120 mins of the IVGTT [mg/dL \times 120 min]; AUC₀₋₁₈₀ = AUC of glucose during the 180 min of the IVGTT [mg/dL \times 180 min]; CR₀₋₃₀ = clearance rate of glucose during the first 30 min of the IVGTT [%/min]; CR₀₋₆₀ = clearance rate of glucose during the first 60 min of the IVGTT [%/min].

^c QUICKI = quantitative insulin sensitivity check index; RQUICKI = revised quantitative insulin sensitivity check index; RQUICKI_{BHB} = revised quantitative insulin sensitivity check index including BHB; HOMA-IR = homeostasis model of insulin resistance.

^d HOMA-IR was log transformed before calculation of Pearson correlation coefficients.

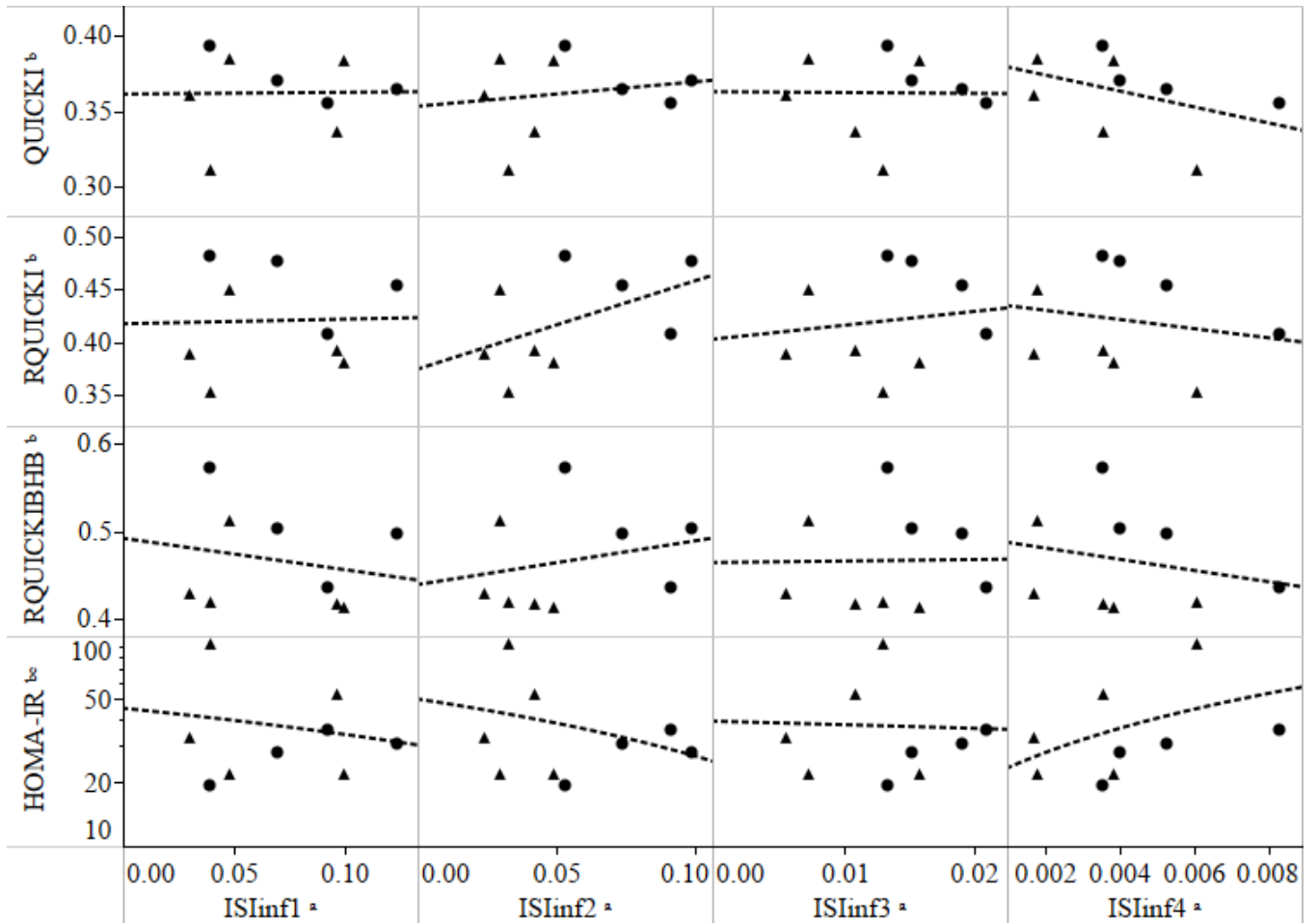


Figure 1. Visualization of the linear relationship between insulin sensitivity indices (ISI)^a derived from the hyperinsulinemic euglycemic clamp (HEC) test and surrogate indices for insulin sensitivity^b. Points (●) represent the individual cows with BCS < 3.75. Triangles (▲) represent the individual cows with BCS > 3.75.

^a ISI derived from the HEC test during each of the 4 insulin infusion periods (ISI_{inf1}, ISI_{inf2}, ISI_{inf3}, ISI_{inf4}) calculated using the following formula $ISI = SSGIR / (\Delta I \times SSG)$, with SSGIR = steady state glucose infusion rate, ΔI = difference between the steady state insulin concentration and the basal insulin concentration and, SSG = steady state glucose concentration, [mL/kg/min per $\mu IU/mL$].

^b QUICKI = quantitative insulin sensitivity check index; RQUICKI = revised quantitative insulin sensitivity check index; RQUICKIBHB = revised quantitative insulin sensitivity check index including BHB; HOMA-IR = homeostasis model of insulin resistance.

^c HOMA-IR was log transformed to yield normal distribution.

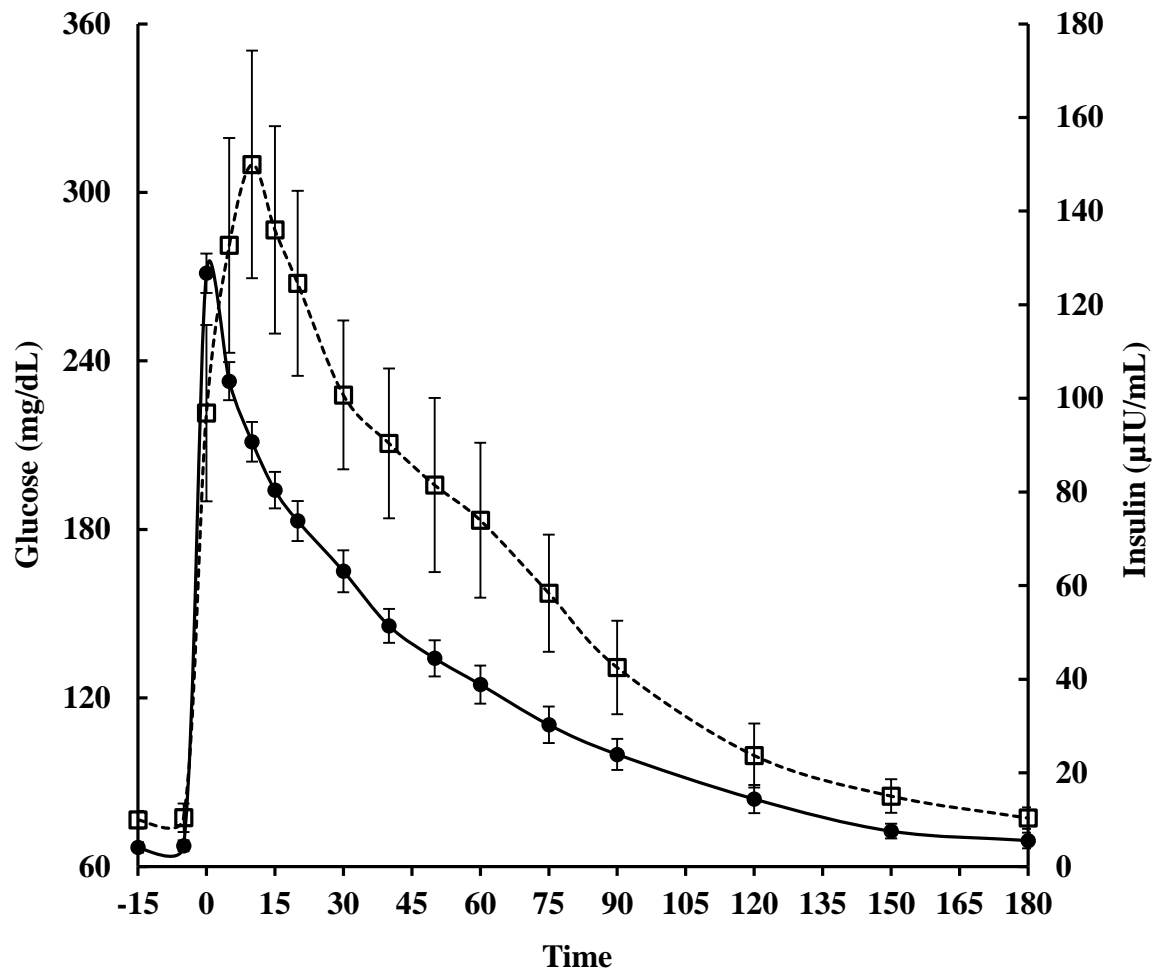


Figure 2. Response of the plasma glucose (●) and serum insulin (□) concentration after an intravenous glucose bolus. Points represent the means of the glucose and insulin concentration, error bars represent the standard error of the mean glucose and insulin concentration.

Clearance rate and AUC derived from the IVGTT (Figure 2) have been frequently used as measures of insulin sensitivity. In the present study, we investigated the CR for the first 30 and 60 min after the intravenous glucose bolus, while AUC was calculated for the first 60 and 120 min and for the total time (180 min) of the IVGTT (Table 5).

Table 5. Measures of insulin sensitivity derived from the intravenous glucose tolerance test (IVGTT).

Measure ^a	Mean \pm SEM
Si	0.817 \pm 0.087
AUC ₀₋₆₀	6290 \pm 315
AUC ₀₋₁₂₀	8367 \pm 558
AUC ₀₋₁₈₀	8818 \pm 642
CR ₀₋₃₀	1.67 \pm 0.087
CR ₀₋₆₀	1.31 \pm 0.062

^a Si = insulin sensitivity derived from the minimal model [$\times 10^{-4}$ ($\mu\text{IU/mL}$)⁻¹ per min];

AUC₀₋₆₀ = area under the curve of glucose during the first 60 min of the IVGTT [mg/dL \times 60 min];

AUC₀₋₁₂₀ = area under the curve of glucose during the first 120 min of the IVGTT [mg/dL \times 120 min];

AUC₀₋₁₈₀ = area under the curve of glucose during the 180 min of the IVGTT [mg/dL \times 180 min];

CR₀₋₃₀ = clearance rate of glucose during the first 30 min of the IVGTT [%/min];

CR₀₋₆₀ = clearance rate of glucose during the first 60 min of the IVGTT [%/min].

No significant correlation existed between CR₀₋₃₀, CR₀₋₆₀ and any of the ISI (Table 4). For the AUC, the relationship was strong, negative and linear between AUC₀₋₆₀ and ISI_{inf2}, while there was a trend for a strong, negative and linear relationship between AUC₀₋₁₂₀, AUC₀₋₁₈₀ and ISI_{inf2} and AUC₀₋₆₀ and ISI_{inf3} (Table 4, Figure 3). The Si derived from the MINMOD (Table 5) and ISI derived from the HEC test (Table 1) were converted to the same units. Concordance correlation coefficients were calculated and Bland-Altman plots were plotted to demonstrate the agreement between Si and ISI (Table 6, Figure 4).

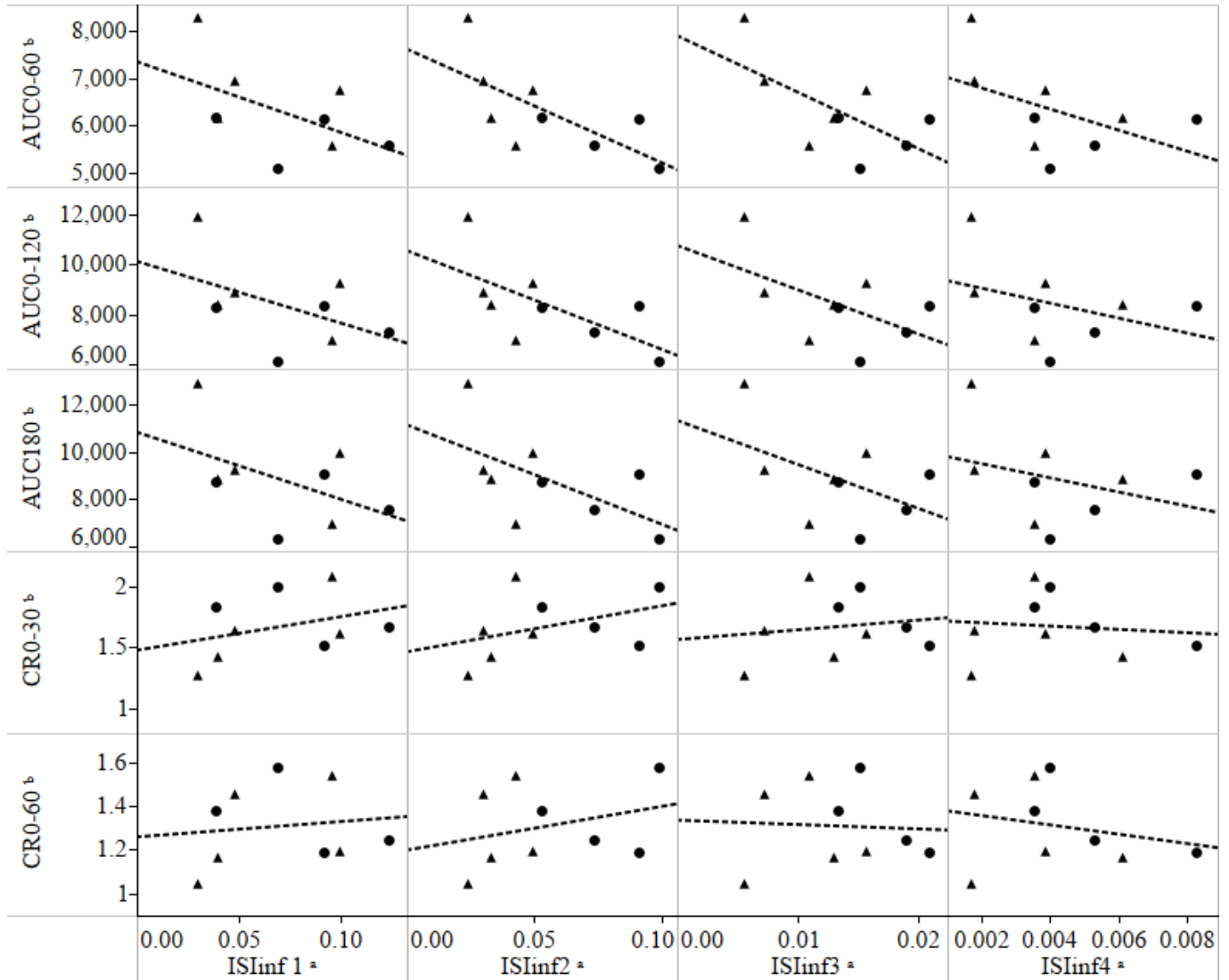


Figure 3. Visualization of the linear relationship between insulin sensitivity indices (ISI)^a derived from the hyperinsulinemic euglycemic clamp (HEC) test and measures of insulin sensitivity derived from the intravenous glucose tolerance test (IVGTT)^b. Points (●) represent the individual cows with BCS < 3.75. Triangles (▲) represent the individual cows with BCS > 3.75.

^aISI derived from the HEC test during each of the 4 insulin infusion periods (ISI_{inf1}, ISI_{inf2}, ISI_{inf3}, ISI_{inf4}) calculated using the following formula $ISI = SSGIR / (\Delta I \times SSG)$, with SSGIR = steady state glucose infusion rate, ΔI = difference between the steady state insulin concentration and the basal insulin concentration and, SSG = steady state glucose concentration, [mL/kg/min per μ IU/mL].

^bAUC₀₋₆₀ = area under the curve (AUC) of glucose during the first 60 min of the IVGTT [mg/dL x 60 min]; AUC₀₋₁₂₀ = AUC of glucose during the first 120 min of the IVGTT [mg/dL x 120 min]; AUC₀₋₁₈₀ = AUC of glucose during the 180 min of the IVGTT [mg/dL x 180 min]; CR₀₋₃₀ = clearance rate of glucose during the first 30 min of the IVGTT [%/min]; CR₀₋₆₀ = clearance rate of glucose during the first 60 min of the IVGTT [%/min].

Table 6. Concordance correlation coefficients with 95% confidence limits between converted values of insulin sensitivity indices (ISI) derived from the hyperinsulinemic euglycemic clamp test^a and converted values of Si derived from MINMOD analysis of the intravenous glucose tolerance test^b

	ISI _{converted,inf 1}	ISI _{converted,inf 2}	ISI _{converted,inf 3}	ISI _{converted,inf 4}
Si _{converted}	0.05	0.04	0.57	0.02
	[-0.18; 0.28]	[-0.18; 0.25]	[0.19; 0.80]	[-0.21; 0.26]

^a Converted ISI calculated using the following formula $ISI_{converted} = ISI \times \text{bodyweight [dL/min per } \mu\text{IU/mL]}$

^b Converted Si derived from the minimal model calculated using the following formula $Si_{converted} = Si \times (\text{glucose dose}) / (G0 - Gb)$; with glucose dose being the total administered dose of glucose in mg, G0 being the postinjection glucose concentration and Gb being the basal glucose concentration derived from the MINMOD [dL/min per $\mu\text{IU/mL}$].

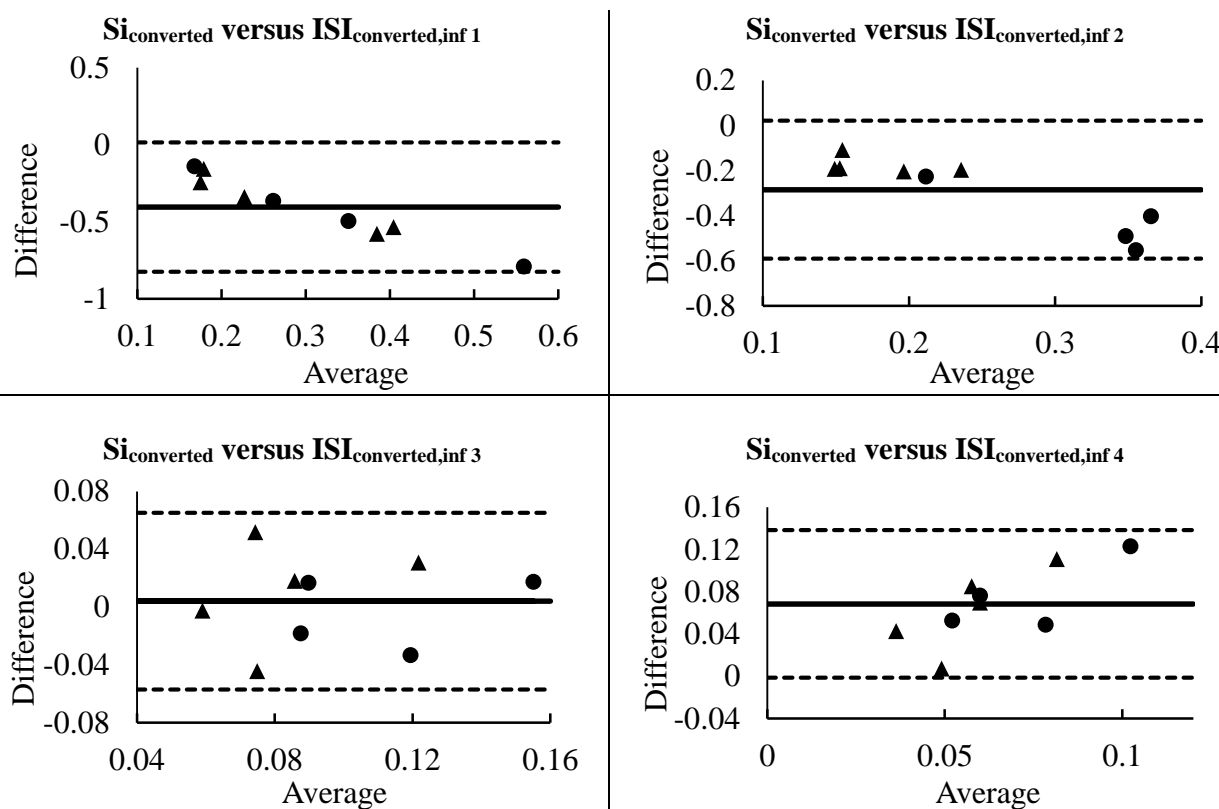


Figure 4. Bland-Altman plots illustrating the agreement between the converted ISI derived from the HEC test and the converted Si derived from the MINMOD analysis of IVGTT. Differences were calculated as $Si_{converted} - ISI_{converted}$. Points (●) represent the individual cows with BCS < 3.75. Triangles (▲) represent the individual cows with BCS > 3.75. Thick line (—) represent the average difference between the two tests, dashed line (- -) represent the 95% confidence limits.

5. DISCUSSION

In the basal state or during dynamic tests, the blood glucose concentration is regulated by multiple hormones and is the result of total body glucose metabolism (Ferrannini and Mari, 1998; De Koster and Opsomer, 2013). The accuracy of a test to measure or describe insulin sensitivity is dependent on the ability of the test to differentiate the effect of insulin on glucose metabolism from all other factors influencing glucose metabolism. Different tests have been proposed to assess the insulin sensitivity of an individual including the HEC test, the IVGTT, the insulin tolerance test and multiple surrogate indices for insulin sensitivity (Muniyappa et al., 2008). While some tests are easy to perform and ideal for epidemiological studies, other tests are more laborious but provide more accurate estimates of insulin sensitivity and should hence be used whenever insulin sensitivity is the main focus of the research (Muniyappa et al., 2008).

Data from the present study are obtained using dairy cows at the end of the dry period, specifically selected to represent a wide range in BCS. In a previous study, we demonstrated that these cows have a large variation in insulin sensitivity of glucose metabolism as measured with the HEC test (De Koster et al., 2015). Hyperinsulinemic euglycemic clamp tests were performed by consecutive administration of four different insulin infusions of increasing dose. The advantage of using consecutive insulin infusions is that steady state conditions are reached earlier compared to single insulin infusion HEC tests without having an impact on the SSGIR (Rizza et al., 1981). Overconditioned cows required less glucose to maintain euglycemia under hyperinsulinemic conditions compared to leaner cows, which indicates increased insulin resistance in the overconditioned cows. This insulin resistant state was characterized by a decreased maximal effect of insulin (decreased insulin responsiveness) and an increased insulin concentration needed to elicit half of its maximal effect (decreased insulin sensitivity) (De Koster et al., 2015). Maximal effect of insulin on glucose metabolism is expected to occur at concentrations obtained by the highest insulin dose during the HEC tests of the present study while half maximal effect is demonstrated to occur at concentrations of 76.41 $\mu\text{IU/mL}$ (De Koster and Opsomer, 2013; De Koster et al., 2015).

Ideally, validation of tests is done by comparison with the gold standard method and by visualization of the differences using Bland-Altman plots and calculating CCC (Dohoo et al., 2003; Carrasco et al., 2013). However, this is only feasible when the values of both tests are given in identical units. Insulin sensitivity indices (ISI) derived from the HEC test can be interpreted as the amount of glucose required to maintain euglycemia per $\mu\text{IU/mL}$ increase of insulin corrected for the basal glucose concentration. For the comparison between ISI from the HEC test and Si from MINMOD analysis of the IVGTT data, Bland-Altman plots and CCC were calculated after conversion of ISI and Si to the same units as described by Bergman et al. (1987). Values obtained from the other tests (CR, AUC, QUICKI, RQUICKI, RQUICK_{BHB}) have different units and the strength and direction of the linear relationship between these tests and the gold standard were assessed by calculating Pearson correlation coefficients as has been done in human medicine (Bonora et al., 2000; Katz et al., 2000; Perseghin et al., 2001).

While in humans, surrogate indices demonstrate a strong relationship with the HEC test (Perseghin et al., 2001; Muniyappa et al., 2008), no significant correlation could be demonstrated between ISI and surrogate indices in cows in the present study. Similarly, other researchers failed to detect strong relationships between surrogate indices and results of IVGTT or ITT (Kerestes et al., 2009; Mann et al., 2016). The lack of correlation in dairy cows may be explained by several factors. First of all, in non-diabetic humans, results obtained by surrogate indices are mainly influenced by the variability in fasting insulin concentration between individuals, due to the compensatory hypersecretion of insulin by the pancreas in an insulin resistant state (Bloomgarden, 2006; Muniyappa et al., 2008). As a consequence, insulin resistant individuals will have elevated insulin and decreased QUICKI, RQUICKI, RQUICK_{BHB} and increased HOMA-IR. In the cows in the present study, very little variability existed in the basal insulin concentration and as a consequence, very little variation was observed in the calculated surrogate indices. The insulin sensitivity and responsiveness of glucose metabolism of the cows in the present study were negatively associated with the accumulation of adipose tissue, as reflected by the BCS (De Koster et al., 2015). However, hyperinsulinemia did not develop in the overconditioned cows in the present study. Similarly, Bradford and Allen (2007) found a weak correlation between BCS and insulin concentration in lactating dairy cows. In the study

by Holtenius et al. (2003), basal insulin concentration in the dry period was affected by feeding level, with cows offered the greatest amount of feed having the highest insulin concentration. Bradford and Allen concluded that the basal insulin concentration might be a reflection of the nutritional status of dairy cows (2007) rather than a reflection of insulin sensitivity. Secondly, in human medicine, the surrogate indices for insulin sensitivity have been calculated based on a single blood sample taken after an overnight fast (Muniyappa et al., 2008). The overnight fasting period in humans elicits a steady state where the glucose and insulin concentration are constant and a reflection of the hepatic and skeletal muscle insulin sensitivity (Muniyappa et al., 2008). In dairy cows, it is impossible to create a fasting state without prolonged starvation, which in turn would provoke compensatory mechanisms affecting glucose, NEFA, BHB and insulin concentrations unrelated to the insulin sensitivity of the animal (Nielsen et al., 2003; Schoenberg et al., 2012). Additionally, diurnal variation and stress associated with sampling influence concentrations of metabolites and might have an important impact on calculated surrogate indices in cows (Allen et al., 2005; Leroy et al., 2011; Mann et al., 2016). Finally, in humans, insulin resistance is associated with a normal or elevated glucose concentration, and occasionally elevated serum triglycerides (Bloomgarden, 2006). Peculiar in dairy cows is the fact that they are in a lactating or pregnant state resulting in a large amount of glucose taken up by the gravid uterus and the lactating mammary gland independent of insulin. Therefore, the use of glucose as an indicator for insulin sensitivity is not straightforward in those physiological states (De Koster and Opsomer, 2013). Despite the fact that NEFA concentrations were associated with insulin resistance in overconditioned cows (De Koster et al., 2015), the inclusion of NEFA and BHB in the calculation of surrogate indices did not improve the correlation with ISI from the HEC test. In dairy cows, NEFA and BHB are used as indicators for the degree of NEB (Ospina et al., 2013) and hence reflect a state of energy deficit rather than a state of insulin resistance.

For the comparison between the HEC test and the IVGTT, the negative relationship between AUC_{0-60} , AUC_{0-120} , AUC_{0-180} and ISI_{inf2} and AUC_{0-60} and ISI_{inf3} is expected. In an insulin sensitive animal, the ISI will be greater due to the fact that more glucose is required per unit of insulin. To the contrary, in the same insulin sensitive animal, the AUC will be less due to the

fact that the clearance of glucose from the blood is greater. A drawback of the AUC as measure of insulin sensitivity is that it only reflects the glucose concentration and does not take into account variations in insulin secretion following a glucose bolus. While the largest variability between cows during the IVGTT was observed in the secretion of insulin. In human medicine, the acute insulin response to glucose is related to the insulin sensitivity (hyperbolic relationship) (Kahn, 2003). Whether the same holds true for cows and which factors influence the insulin secretory capacity of the pancreas in periparturient cows, warrants further research.

The S_i derived from the MINMOD can be interpreted as the effect of insulin to increase the disappearance of glucose (Bergman et al., 1979). The S_i values of the present study are comparable with dairy cows at the end of pregnancy (Salin et al., 2012), somewhat lower compared to nonlactating and nonpregnant animals (Pires et al., 2007) but much lower than values from lactating dairy cows (Back et al., 2007; Maret et al., 2015). During lactation, it is expected that large amounts of glucose are taken up by the mammary gland independent of insulin (De Koster and Opsomer, 2013). Additionally, insulin secretion is lower in lactating cows compared to cows at the end of the dry period (Holtenius et al., 2003; Bossaert et al., 2008; Kerestes et al., 2009). Therefore, S_i values derived from the MINMOD analysis might overestimate insulin sensitivity in lactating cows and more research is needed to determine the impact of the glucose uptake by the mammary gland on estimates of insulin sensitivity in lactating dairy cows.

Finegood et al. (1984), Beard et al. (1986) and Bergman et al. (1987) validated the minimal model to be used in humans and dogs and found good correlations between insulin sensitivity derived from the HEC test and the IVGTT. In the present study, a moderate agreement was found between S_i and ISI_{inf3} , while S_i underestimates insulin sensitivity as measured by ISI_{inf1} and ISI_{inf2} and overestimates ISI_{inf4} . Thus S_i can be interpreted as a fair estimate of insulin sensitivity but not insulin responsiveness. The advantage of the MINMOD analysis is that it fits the glucose curve of an animal based on the insulin secretory capacity of the pancreas and therefore, it gives a more reliable estimate of insulin sensitivity compared to AUC.

6. CONCLUSIONS

Surrogate indices for insulin sensitivity (HOMA-IR, QUICKI, RQUICKI and RQUICKI_{BHB}) are not associated with measures of insulin sensitivity derived from the HEC test in dairy cows at the end of the dry period. Comparisons between measures derived from IVGTT and HEC tests indicate that AUC₀₋₆₀ and Si derived from MINMOD analysis of the IVGTT are associated with measures of insulin sensitivity derived from the HEC test. The Si derived from MINMOD analysis of the IVGTT is expected to be more reliable than AUC₀₋₆₀ due to the fact that it integrates the insulin secretion in the prediction of the insulin sensitivity parameter. It should be emphasized that results from the present study relate to dairy cows at the end of the dry period. Due to drastic homeorhetic changes at the beginning of lactation, especially of the glucose, insulin and NEFA metabolism, more research is needed to identify reliable measures of insulin sensitivity at the beginning of lactation and during the remainder of the lactation.

7. ACKNOWLEDGMENTS

This research was funded by the Special Research Fund of Ghent University, grant number 01D28410. The authors wish to thank Lars Hulpio, Isabel Lemahieu and Petra Van Damme for excellent technical assistance in the lab.

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CHAPTER 4.2

Limitations of glucose tolerance tests in the assessment of peripheral tissue insulin sensitivity during pregnancy and lactation in dairy heifers

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2016. Limitations of glucose tolerance tests in the assessment of peripheral tissue insulin
sensitivity during pregnancy and lactation in dairy heifers. Journal of Dairy Science
(Accepted).*

1. ABSTRACT

The aim of the present study was to point at the limitations of glucose tolerance tests to assess peripheral tissue insulin sensitivity in dairy heifers in different physiological states (pregnancy and lactation). Intravenous glucose tolerance tests (GTT) were performed in five nonpregnant-nonlactating heifers, five heifers at the end of pregnancy (12 to 7 days before calving) and five lactating primiparous cows (11 to 14 days after calving). Glucose and insulin concentrations were determined and area under the curve (AUC) and clearance rate (CR) of glucose and insulin were calculated. Additionally, data were analyzed using the minimal model to derive the insulin sensitivity parameter (S_i). Basal glucose and insulin concentrations were greater in the nonpregnant-nonlactating heifers. Clearance rate of glucose and S_i were lowest while the AUC for glucose was greatest in the nonpregnant-nonlactating heifers. Insulin concentrations during the GTT were greater for the nonpregnant-nonlactating heifers. Results from the GTT in pregnant heifers and lactating primiparous cows are biased by the fact that a large part of the glucose disappearance during an intravenous GTT occurs independently of insulin by the pregnant uterus or the lactating mammary gland. As such, greater AUC of glucose, lower CR or lower S_i derived from GTT performed in nonpregnant-nonlactating dairy heifers in the present study might indicate decreased peripheral tissue insulin sensitivity of the glucose metabolism or decreased insulin independent glucose disappearance. Based on the results from a GTT, it is impossible to discriminate between both metabolic pathways. It can be concluded that parameters derived from GTT are not indicated to compare peripheral tissue insulin sensitivity of the glucose metabolism between dairy heifers in different physiological states due to the large variation in insulin secretion and the substantial difference in insulin independent glucose disposal associated with these physiological states.

2. INTRODUCTION

Glucose tolerance tests (GTT) are frequently used to assess insulin sensitivity in dairy cows (Holtenius et al., 2003; Chagas et al., 2009). The plasma glucose profile during a GTT is the reflection of total body glucose metabolism after an intravenous glucose bolus (Ferrannini and Mari, 1998; De Koster and Opsomer, 2013). The total body glucose metabolism can be subdivided in the insulin independent and insulin dependent glucose metabolism. The insulin

dependent glucose metabolism is influenced by the increase in insulin concentration after an intravenous glucose bolus and stimulates insulin sensitive tissues (mainly skeletal muscle and to a lesser extent adipose tissue) to increase GLUT4 translocation to the plasma membrane followed by a subsequent increase in glucose uptake. The response of insulin sensitive tissues to insulin determines the insulin resistance of the animal (Kahn, 1978; De Koster and Opsomer, 2013).

The insulin independent glucose metabolism is not influenced by the increased insulin concentration during the GTT, but is mainly determined by the capacity of an increased glucose concentration to enhance its own disappearance and to inhibit hepatic glucose output (Bergman, 2007; Muniyappa et al., 2008). In nonpregnant-nonlactating animals, the insulin independent glucose disappearance occurs mainly via GLUT1 in different tissues and by excretion via the kidney (only small amounts, 2 to 4 % of the glucose bolus) (Grunberg et al., 2011). In pregnant animals, a large part of the circulating glucose (60 to 70%) is taken up independently of insulin by the pregnant uterus via GLUT1 and GLUT3 (Bell et al., 2000; Grunberg et al., 2011). This is even more pronounced in lactating animals, in which the insulin independent glucose uptake by the udder via GLUT1 increases glucose requirements substantially (De Koster and Opsomer, 2013). In lactating cows, insulin independent glucose uptake by the udder was estimated to be responsible for 80% to 82% of whole body glucose turnover (Bickerstaffe et al., 1974; Bell and Bauman, 1997; Rose et al., 1997).

The ability of a test to assess the insulin sensitivity of the glucose metabolism is dependent on its ability to differentiate between the effect of insulin to enhance glucose disappearance and all other factors influencing glucose disappearance (De Koster et al., 2016). Different tests have been used to assess insulin resistance in dairy cows. Surrogate indices for insulin sensitivity (calculated from glucose, insulin, NEFA and BHB concentrations in blood) were unreliable to assess insulin resistance in dairy cows at the end of the dry period, due to inherent differences in metabolism between humans and ruminants. In humans, insulin resistance is characterized by high insulin and/or high glucose concentrations while the metabolism of dry and lactating cows is characterized by low glucose and low insulin concentrations (Bloomgarden, 2006; De Koster and Opsomer, 2013; De Koster et al., 2016). In humans, elevated concentrations of

triglycerides have been associated with insulin resistance (Bloomgarden, 2006). While, NEFA and BHB in dairy cows are reflections of negative energy balance rather than a state of insulin resistance (Ospina et al., 2013; De Koster et al., 2016). Parameters derived from GTT (area under the curve for glucose, insulin sensitivity index derived from the minimal model) were proven to be reliable estimates of insulin resistance in dairy cows at the end of the dry period (De Koster et al., 2016). Dairy cows generally are in a lactating and/or pregnant state, leading to a large part of the glucose disappearance being insulin independent (60 to 82%) (Rose et al., 1997; De Koster and Opsomer, 2013) and making it difficult to interpret and compare parameters derived from GTT performed in cows in different physiological states (Marett et al., 2015). In literature however, multiple papers can be found, in which the GTT has been used to measure and compare insulin resistance in cows in different physiological states without taking into account the potential difference in insulin independent glucose disappearance or differences in glucose induced insulin secretion (Chalmeh et al., 2015; Oliveira et al., 2016). The aim of the present study was to point at the limitations of glucose tolerance tests to assess peripheral tissue insulin sensitivity in dairy heifers in different physiological states (pregnancy and lactation). We hypothesized that the results from GTT would differ depending on the physiological state of the animals but interpretation of the parameters derived from GTT would be indefinite in terms of insulin resistance.

3. MATERIALS AND METHODS

All experimental procedures were approved by the ethical committee of the Faculty of Veterinary Medicine (EC2015/142 - Ghent University, Belgium). Intravenous GTT were performed in nonpregnant-nonlactating heifers (NON, n = 5), pregnant-nonlactating heifers 12 to 7 days before calving (PREG, n = 5) and nonpregnant-lactating primiparous cows 11 to 14 days after calving (LACT, n = 5). On the day of the GTT, heifers were weighed, BCS was assessed according to the scale of Edmonson et al. (1989), and backfat thickness (BFT) was determined as described by Schröder and Staufenbiel (2006). A catheter (Cavafix Certo 338 - 14G, B. Braun, Instrulife, Oostkamp, Belgium) was placed in the left jugular vein and heifers were allowed to rest for a period of 2 h. Glucose was infused at a dose of 150 mg/kg body weight (Glucose 30%, Eurovet, Verdifarm, Beringen-Paal, Belgium) over a period of 2 to 4

min, after which the catheter was flushed two times with 20 mL of saline. Time point 0 was the moment when all the glucose was infused. Blood samples for determination of glucose and insulin were taken at following time points: -15, -5, 0, 2, 4, 6, 8, 10, 12, 15, 18, 20, 23, 26, 30, 35, 40, 45, 50, 60, 90, 120, 150 and 180 min. From 2 h before the GTT until the end, heifers had access to fresh drinking water but not feed.

Samples for plasma glucose determination were taken in fluoride blood tubes (Vacutest, Novolab, Geraardsbergen, Belgium). Samples for serum insulin determination were taken in gel-coated blood tubes (Vacutest, Novolab, Geraardsbergen, Belgium). Within 2 h after collection, all blood samples were centrifuged for 20 min ($2,460 \times g$, 7°C) and serum and plasma were stored at -20°C until analysis. Plasma glucose concentrations were determined using a colorimetric hexokinase method on a Cobas 6000 analyzer (Roche diagnostics, Mannheim, Germany), intra- and inter-assay CV were 0.82% and 1.1%, respectively. Serum insulin concentrations were determined using a bovine specific commercial ELISA kit (Bovine Insulin ELISA, Catalog nr 10-1201-01, Mercodia, Uppsala, Sweden), intra- and inter-assay CV were 2.9% and 10.8%, respectively. Conversion of insulin concentrations from gravimetric units to international units was done as described by Abuelo et al. (2012).

Based on the measured glucose and insulin concentrations during the GTT, different measures of insulin sensitivity were calculated. The clearance rate of glucose between 0 and 30 min ($\text{CR}_{0-30,\text{glucose}}$) and 0 and 60 min ($\text{CR}_{0-60,\text{glucose}}$) and the clearance rate of insulin between 15 and 30 min ($\text{CR}_{15-30,\text{insulin}}$) were calculated as described by Pires et al. (2007). The area under the curve (AUC) of glucose and insulin were calculated between 0 and 60 min ($\text{AUC}_{0-60,\text{glucose}}$ and $\text{AUC}_{0-60,\text{insulin}}$), between 0 and 120 min ($\text{AUC}_{0-120,\text{glucose}}$ and $\text{AUC}_{0-120,\text{insulin}}$) and between 0 and 180 min ($\text{AUC}_{0-180,\text{glucose}}$ and $\text{AUC}_{0-180,\text{insulin}}$) as the incremental AUC using the trapezoidal rule as described by Cardoso et al. (2011). Glucose and insulin data derived from the GTT were fitted using the MINMOD Millenium software (MINMOD Inc., Pasadena, CA) (Boston et al., 2003) based on the minimal model as described by Bergman et al. (1979). The derived variables of interest were the insulin sensitivity index or Si and the glucose effectiveness or Sg . The insulin sensitivity index is a measure describing the ability of insulin to increase glucose disappearance. The glucose effectiveness is a measure describing the ability of glucose to enhance its own

disappearance, independent of insulin (Bergman et al., 1979). For model fitting purposes in the MINMOD program, the results of the GTT were adapted as follows: basal glucose and insulin concentrations measured at time -15 and -5 min were averaged and assigned to time point 0 min in the MINMOD program; insulin and glucose concentrations measured at time point 0 min were assigned to time point 2 min in the MINMOD program; results from the first 8 min during the GTT were zero-weighted to allow for glucose mixing and basal values for glucose and insulin were added at time points 240 and 300 min (Bergman et al., 1979; Pacini and Bergman, 1986; Boston et al., 2003). The curves generated by the MINMOD program were evaluated by visual assessment of the fit of the curves with the original data, the fractional standard deviation ($< 15\%$) and the coefficient of determination ($r^2 > 95\%$) (Pacini and Bergman, 1986).

Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Descriptive statistics (PROC MEANS) are expressed as mean \pm SEM unless otherwise indicated. Normality of the variables (PROC UNIVARIATE) was checked using the Kolmogorov-Smirnov test. All variables were compared in an ANOVA model with group as independent variable (3 categories: NON, PREG and LACT). Homoscedasticity was checked using the Levene's test. When variances within groups differed significantly, variables were \log_{10} transformed. Conclusions made after log transformation of the data were similar compared with the non-transformed data. Pair-wise comparisons were made between groups using the Tukey honest significant difference test. Differences between groups were declared significant at $P < 0.05$.

4. RESULTS AND DISCUSSION

The age, BCS, BFT, weight and realized 305 d milk production of the heifers are given in Table 1. Pregnant heifers were heavier compared with the two other groups most probably due to the weight of the fetus, uterus, amniotic and allantoic fluids. Fatter cows and heifers have lower insulin sensitivity (McCann and Reimers, 1985; De Koster et al., 2015). In the present study, the degree of fatness as measured by the BCS and BFT did not differ between the groups. The NON heifers, although at an average age over 27 months, had not been bred due to the fact that they had been used for ovum pick up.

Table 1: Age, weight, BCS, BFT and realized 305 d milk production of the nonpregnant-nonlactating heifers (NON), pregnant heifers (PREG) and lactating primiparous cows (LACT) (mean \pm SEM).

	NON	PREG	LACT	<i>P</i> -value ⁵
Age (months) ¹	27.8 \pm 3.2	23.5 \pm 0.6	24.2 \pm 0.7	0.283
Weight (kg)	484.2 \pm 20.0 ^a	579.6 \pm 8.9 ^b	523.8 \pm 8.9 ^a	0.001
BCS ²	2.70 \pm 0.15	3.10 \pm 0.17	3.00 \pm 0.16	0.218
BFT (cm) ³	0.87 \pm 0.09	0.96 \pm 0.19	1.10 \pm 0.17	0.591
305 d milk production (kg) ⁴	-	7,838 \pm 268	7,510 \pm 557	0.609

^{ab} Means within a row with different superscripts differ (Tukey post hoc test, $P < 0.05$).

¹ Age at the moment of the glucose tolerance test;

² Body condition score according to the scale of Edmonson et al. (1989);

³ Backfat thickness as described by Schröder and Staufenbiel (2006);

⁴ Realized 305 d milk production;

⁵ *P* values of the ANOVA for group differences.

Basal glucose and insulin concentration were greater in the NON group compared to the PREG and LACT group (Table 2). Following the glucose infusion, peak glucose concentration was greater in the NON group most probably due to the greater basal glucose concentration and the lower glucose requirements (Figure 1, Table 2). While the AUC_{glucose} was larger for every calculated interval in the NON group, the CR_{glucose} was lower only for the interval between 0 and 30 min. Insulin secretion, as measured by peak insulin concentration and AUC_{insulin}, was greater in the NON group, followed by the PREG group, with the lowest insulin secretion in the LACT group (Figure 1, Table 2). Whole body response to a glucose load in dairy heifers can be partitioned into insulin secretion by the pancreas and insulin dependent and independent glucose disappearance.

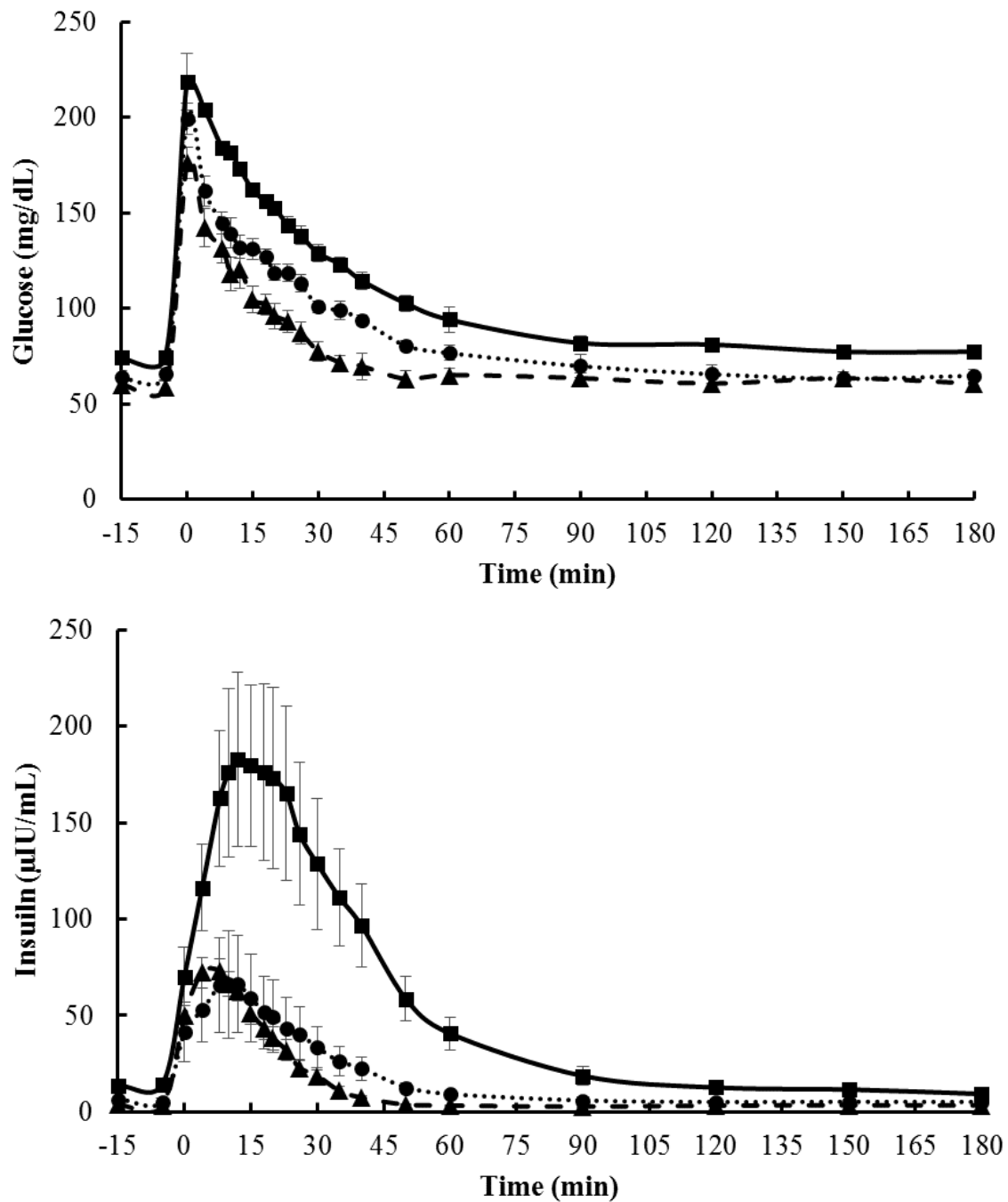


Figure 1: Glucose (mg/dL) and insulin concentration (μ IU/mL) during the intravenous glucose tolerance test in 5 nonpregnant-nonlactating heifers (—■—), in 5 pregnant heifers (··●··) and in 5 lactating primiparous cows (—▲—). Symbols represent the average glucose and insulin concentration at each time point, error bars represent the standard error of the mean.

Table 2: Results of the ANOVA for group differences for the parameters derived from the glucose tolerance tests (GTT) performed in nonpregnant-nonlactating heifers (NON), pregnant heifers (PREG) and lactating primiparous cows (LACT) (mean \pm SEM).

	NON	PREG	LACT	<i>P</i> - value
Basal glucose (mg/dL) ¹	74.3 \pm 1.9 ^a	64.5 \pm 3.4 ^b	59.5 \pm 2.92 ^b	0.009
Basal insulin (μ IU/mL) ²	14.11 \pm 1.49 ^a	5.91 \pm 1.98 ^b	3.39 \pm 0.65 ^b	0.001
Peak glucose (mg/dL) ³	203 \pm 6.0 ^a	163 \pm 9.0 ^b	153 \pm 11.0 ^b	0.005
Peak insulin (μ IU/mL) ⁴	186.6 \pm 44.9 ^a	70.7 \pm 26.7 ^b	76.5 \pm 7.1 ^{ab}	0.026
CR _{0-30,glucose} (%/min) ⁵	1.73 \pm 0.25 ^a	2.26 \pm 0.08 ^{ab}	2.75 \pm 0.20 ^b	0.010
CR _{0-60,glucose} (%/min) ⁵	1.40 \pm 0.18	1.59 \pm 0.09	1.66 \pm 0.08	0.353
CR _{15-30,insulin} (%/min) ⁶	2.42 \pm 0.35 ^a	3.61 \pm 1.15 ^a	6.83 \pm 0.40 ^b	0.003
AUC _{60glucose} (mg/dL x 60 min) ⁷	3,875 \pm 184 ^a	2,802 \pm 172 ^b	1,860 \pm 158 ^c	< 0.001
AUC _{120glucose} (mg/dL x 120 min) ⁷	4,508 \pm 367 ^a	3,147 \pm 397 ^b	2,082 \pm 202 ^b	0.001
AUC _{180glucose} (mg/dL x 180 min) ⁷	4,751 \pm 357 ^a	3,117 \pm 489 ^b	2,238 \pm 235 ^b	0.002
AUC _{60insulin} (μ IU/mL x 60 min) ⁸	6,120 \pm 1,528 ^a	1,759 \pm 567 ^b	1,505 \pm 162 ^b	0.004
AUC _{120insulin} (μ IU/mL x 120 min) ⁸	6,636 \pm 1,669 ^a	1,793 \pm 556 ^b	1,495 \pm 163 ^b	0.003
AUC _{180insulin} (μ IU/mL x 180 min) ⁸	6,471 \pm 1,664 ^a	1,745 \pm 536 ^b	1,496 \pm 177 ^b	0.004
Si ($\times 10^{-4}$ (μ IU/mL) ⁻¹ per min) ⁹	0.95 \pm 0.29 ^a	3.99 \pm 1.37 ^b	3.83 \pm 0.43 ^b	0.005
Sg (/min) ¹⁰	0.017 \pm 0.003	0.021 \pm 0.004	0.027 \pm 0.008	0.424

^{abc} Means within a row with different superscripts differ (Tukey post hoc test, $P < 0.05$).

¹ Average glucose concentration in blood samples taken before the start of the GTT;

² Average insulin concentration in blood samples taken before the start of the GTT;

³ Glucose concentration at time 0 as derived from the MINMOD;

⁴ The maximum insulin concentration measured during the GTT;

⁵ Clearance rate of glucose during the first 30 min and 60 min of the GTT;

⁶ Clearance rate of insulin between 15 and 30 min of the GTT;

⁷ Area under the curve of glucose during the first 60 min, the first 120 min and 180 min of the GTT;

⁸ Area under the curve of insulin during the first 60 min, the first 120 min and 180 min of the GTT;

⁹ Insulin sensitivity index derived from the minimal model;

¹⁰ Glucose effectiveness derived from the minimal model.

4.1. Glucose induced insulin secretion

For the interpretation of parameters derived from GTT, one needs first to consider the difference in glucose induced insulin secretion by the pancreas between the groups (Ferrannini and Mari, 1998). Following the glucose bolus, insulin concentration increased more in NON heifers. In every group there was a large variation in insulin secretion between the animals, especially in the NON and the PREG group. The large variability of the serum insulin concentration between animals during a GTT has been observed previously by others (Marett et al., 2015). Serum insulin concentrations are influenced by the insulin sensitivity and the physiological state of the animal. From human medicine, it is known that as a compensation mechanism to guarantee glucose homeostasis, insulin secretion is greater in insulin resistant individuals and pregnant women (Kahn, 2003; Buchanan et al., 2012). In case the pancreas is unable to increase its insulin secretion, type 2 diabetes mellitus or gestational diabetes mellitus will develop (Kahn, 2003; DeFronzo, 2004; Buchanan et al., 2012). Due to the large difference in glucose metabolism between ruminants and monogastrics (De Koster and Opsomer, 2013), it is currently unknown whether the same hyperbolic relationship between peripheral tissue insulin resistance and insulin secretion is evident in dairy cows as well.

In dairy cows, it is known that insulin secretion is decreased at the end of pregnancy and the initiation of lactation. This is a normal phenomenon supporting the metabolic need to redirect glucose from peripheral tissues towards the uterus and mammary gland in order to support the growing fetus or nursing neonate (Ingvarsen and Andersen, 2000; Bossaert et al., 2008). Additionally, liver blood flow and metabolic activity increase tremendously in cows at the beginning of lactation, which might contribute to lower insulin levels due to its greater metabolic clearance by the liver (Mann et al., 2016) as demonstrated by the greater CRinsulin in the LACT group (Table 2). Another possible interpretation of our results could be that the glucose induced insulin secretion was lower in the LACT and PREG group due to the lower peak glucose which might have been due to the flux of glucose towards the mammary gland and the uterus. In the present study, no significant differences in AUCinsulin were observed between the PREG and LACT group, although the LACT group had numerically lower AUCinsulin (Table 2). In other studies using multiparous cows, greater glucose induced insulin

secretion by the pancreas was measured in the prepartum compared with the postpartum period (Holtenius et al., 2003; Kerestes et al., 2009; Mann et al., 2016; Weber et al., 2016). The difference with our study might be explained by the following factors. First of all, insulin secretion following a glucose bolus in the prepartum period, decreases as calving approaches (Regnault et al., 2004; Mann et al., 2016). Therefore, the close timing to calving in the present study (12 to 7 days before calving and 11 to 14 days after calving) might limit the distinction between the pre- and postpartum insulin secretion. Secondly, the low number of animals in the different groups might have limited the distinction between the PREG and LACT group. Finally, metabolic differences between heifers and cows may influence parameters derived from GTT. Not only due to the inherently lower milk yield in heifers but also due to the requirements of metabolites for continued growth while pregnant or lactating (NRC, 2001). Adolescent animals prioritize growth before offspring (Wallace et al., 1996; Wallace et al., 1997). Further research is needed to elucidate the difference in glucose and insulin metabolism between growing heifers and cows in the periparturient period.

4.2. Insulin resistance

To interpret the results in terms of insulin resistance, it is important to collectively evaluate the glucose and insulin curves. The AUC_{glucose} and AUC_{insulin} were significantly greater in the NON group followed by the PREG group and being lowest in the LACT group. These results can be interpreted such that the NON group experienced the greatest peripheral tissue insulin resistance since they required more insulin (higher dose) while having a greater AUC_{glucose} (lower response). On the other hand, the LACT group had a lower insulin secretion (lower dose) while having a lower AUC_{glucose} (greater response), which could be interpreted as the lowest peripheral tissue insulin resistance. Using the MINMOD, the glucose response is mathematically predicted using the insulin concentrations after the glucose bolus, thus accounting for differences in insulin secretion between animals (Ferrannini and Mari, 1998). The results of the MINMOD indicate a lower insulin sensitivity in the NON group (low S_i) in comparison with the LACT and PREG group (high S_i) (Table 2). However, this is in contrast to what has generally been accepted in dairy cows: at the end of pregnancy and in the beginning of lactation, these animals are stated to be in a transient state of insulin resistance (Bell and

Bauman, 1997). Hence, the parameters derived from the GTT in the present study seem to be biased by the fact that the insulin independent glucose uptake is different according to the physiological state of the animals. In the LACT group, a large part of the infused glucose is redirected towards the mammary gland independent of insulin (Marett et al., 2015). This is associated with a faster decline of glucose (CR0-30,glucose), a lower AUC of glucose and numerically higher Sg during the GTT. In the PREG group, a large part (however smaller compared to the part going to the mammary gland in the LACT group) of the infused glucose is redirected towards the pregnant uterus independent of insulin. While in the NON group, most of the glucose is disposed in an insulin dependent way. The difference in insulin independent glucose disposal might explain a large part of the observed difference in AUCglucose, CRglucose and Si between groups and might therefore influence the accurate interpretation of the parameters derived from GTT with regard to the insulin sensitivity of the individuals. Other methods, like the hyperinsulinemic euglycemic clamp test using isotopes of glucose, are more suitable for the assessment of insulin dependent and independent glucose disappearance and to compare the insulin sensitivity of dairy heifers and cows in different physiological states (Rose et al., 1997; Weber et al., 2016).

5. CONCLUSION

In conclusion, the effect of physiological state on parameters derived from GTT in the present study indicate improved glucose tolerance in pregnant heifers and lactating primiparous cows compared with nonpregnant-nonlactating heifers. The latter observation might indicate increased peripheral tissue insulin sensitivity of the glucose metabolism or increased insulin independent glucose disappearance in pregnant heifers and lactating primiparous cows (Marett et al., 2015). Based on the results from GTT, it is impossible to discriminate between both metabolic pathways. Since insulin independent glucose disappearance is a very important phenomenon in pregnant and lactating animals, this cannot be ignored in the interpretation of the parameters derived from GTT. As such, parameters derived from GTT are not indicated to compare peripheral tissue insulin sensitivity of the glucose metabolism between dairy heifers (and cows) in different physiological states (pregnant versus lactating versus nonpregnant-

nonlactating). The underlying reason for this is the large variation in insulin secretion and the difference in insulin independent glucose disposal between these physiological states.

6. ACKNOWLEDGMENTS

This research was funded by the Special Research Fund of Ghent University, grant number 01D28410. The authors wish to thank Lars Hulpio, Isabel Lemahieu and Petra Van Damme for excellent technical assistance in the lab.

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CHAPTER 5

Insulin response of the glucose and fatty acid metabolism in dry dairy cows across a range of body condition scores

De Koster J., Hostens M., Van Eetvelde M., Hermans K., Moerman S., Bogaert H., Depreester E., Van den Broeck W. and Opsomer G. 2015. Insulin response of the glucose and fatty acid metabolism in dry dairy cows across a range of body condition score. Journal of Dairy Science. 98:4580-4592.

1. ABSTRACT

The objective of the present research was to determine the insulin response of the glucose and fatty acid metabolism in dry dairy cows with a variable body condition score (BCS). Ten pregnant Holstein Friesian dairy cows (upcoming parity 2 to 5) were selected based on BCS at the beginning of the study (2 months before expected parturition date). During the study, animals were weekly monitored for BCS and backfat thickness and in the last 2 weeks, blood samples were taken for determination of serum non-esterified fatty acid (NEFA) concentration. Animals underwent a hyperinsulinemic euglycemic clamp test in the third week before the expected parturition date. The hyperinsulinemic euglycemic clamp test consisted of 4 consecutive insulin infusions with increasing insulin dose: 0.1; 0.5; 2; and 5 mIU/kg/min. For each insulin infusion period, a steady state was defined as a period of 30 minutes where no or minor changes of the glucose infusion were necessary to keep the blood glucose concentration constant and near basal levels. During the steady state, the glucose infusion rate (steady state glucose infusion rate (SSGIR) in $\mu\text{mol/kg/min}$) and NEFA concentration (steady state NEFA concentration (SSNEFA) in mmol/L) were determined and reflect the insulin response of the glucose and fatty acid metabolism. Dose response curves were created based on the insulin concentrations during the steady state and the SSGIR or SSNEFA. The shape of the dose response curves is determined by the concentration of insulin needed to elicit the half maximal effect (EC50) and the maximal SSGIR or the minimal SSNEFA for the glucose or fatty acid metabolism, respectively. The maximal SSGIR was negatively associated with variables reflecting adiposity of the cows (BCS, backfat thickness, NEFA concentration during the dry period, and absolute weight of the different adipose depots determined after euthanasia and dissection of the different depots), while the EC50 of the glucose metabolism was positively associated with these variables. These results reflect a decreased insulin sensitivity and a decreased insulin responsiveness of the glucose metabolism in overconditioned dry dairy cows. The minimal SSNEFA and the EC50 of the fatty acid metabolism were not associated with variables reflecting adiposity of the cows, meaning that the insulin response of the fatty acid metabolism was not associated with the level of fat accumulation in dry dairy cows. Additionally, within individual cows, the EC50 of the glucose metabolism was higher than the

EC50 of the fatty acid metabolism, meaning that the response of the fatty acid metabolism occurs at lower insulin concentrations compared to the response of the glucose metabolism. It can be concluded that there is a negative association between the level of fat accumulation in pregnant dairy cows at the end of the dry period and the insulin response of the glucose metabolism.

2. INTRODUCTION

Insulin is an important hormone in the metabolism of mammals, birds, reptiles, fish, and amphibians. The metabolic effect of insulin is regulated by two factors. First, the secretory capacity of the β cells of the pancreas which determines the amount of insulin circulating in the blood stream. Second, the binding of insulin to its receptor and the activation of the intracellular signaling cascade in the insulin-sensitive tissues which determines the insulin response of a certain tissue. Insulin resistance is defined as a state where a normal concentration of insulin induces a reduced biological response in the insulin-sensitive tissues. If the maximal effect of insulin is decreased, this is known as decreased insulin responsiveness. If more insulin is needed to elicit the half maximal effect, this is known as decreased insulin sensitivity. Insulin resistance can be attributed to a decrease in insulin responsiveness, insulin sensitivity, or both (Kahn, 1978).

In most mammals, and in dairy cows as well, the end of pregnancy and the initiation of lactation are accompanied by a transient state of decreased insulin action at the level of the glucose metabolism. This homeorhetic adaptation serves as a mechanism to preserve sufficient glucose supply for the gravid uterus and the lactating mammary gland in support of the growing offspring (Bell and Bauman, 1997; De Koster and Opsomer, 2013). Whereas this transient state of decreased insulin action in the periparturient period is considered a normal physiological phenomenon, excessive derailment of insulin's action in peripheral tissues is known to contribute in several pathological disorders in both human and veterinary medicine.

In human medicine, insulin resistance is a central disorder in the metabolic syndrome, a term used to group several obesity associated disorders like type 2 diabetes mellitus, hypertension, and cardiovascular disease. In the development of the metabolic syndrome, the obese adipose tissue plays a detrimental role by the release of NEFA and the production of a variety of

adipokines (Wajchenberg, 2000; Cornier et al., 2008). Similar to obesity in human medicine, overconditioning in dairy cows is an important risk factor for the development of metabolic, digestive, infectious, and reproductive problems in the immediate post-partum period (Morrow, 1976; Roche et al., 2013). The increased susceptibility towards a variety of diseases in overconditioned cows may be explained by excessive NEFA mobilization due to a more pronounced negative energy balance (Grummer et al., 2004). Additionally, the adipose tissue of overconditioned cows has an increased sensitivity towards lipolytic signals and a decreased sensitivity towards antilipolytic signals (Rukkwamsuk et al., 1998; Rukkwamsuk et al., 1999; Kokkonen et al., 2005). Although it has been suggested that impaired insulin action at the level of the glucose and fatty acid metabolism is an important contributor in the excessive mobilization of NEFA in overconditioned cows and the development of periparturient problems (Holtenius et al., 2003; Holtenius and Holtenius, 2007; De Koster and Opsomer, 2013), clear evidence for impaired insulin action at the level of the glucose and fatty acid metabolism in clinically healthy overconditioned dairy cows does not exist.

The present study was conducted in dairy cows selected based on BCS at the end of the dry period. The aim of the study was to determine the insulin response of the glucose and fatty acid metabolism of dairy cows with a variable BCS using the hyperinsulinemic euglycemic clamp (HEC) test. Because of the importance of the visceral adipose tissue in the development of insulin resistance in humans (Wajchenberg, 2000), we determined the amount of fat in the different adipose depots by dissection of adipose tissue from subcutaneous, abdominal, intrapelvic and intrathoracal depots. Next, we aimed to determine the influence of the weight of the different adipose depots and the proportional distribution of adipose tissue on the insulin response of the glucose and fatty acid metabolism.

3. MATERIALS AND METHODS

All experimental procedures were approved by the ethical committee of the Faculty of Veterinary Medicine (EC2010/149 – Ghent University, Belgium).

3.1. Study design

Ten clinically healthy, pregnant Holstein Friesian dairy cows (upcoming parity 2 to 5) were studied from 2 months before the expected parturition date. At the beginning of the study, animals were selected based on BCS according to the scale of Edmonson et al. (1989) to assure an equal spread in BCS from normal conditioned (2.5 - 3.5; n=5) to overconditioned (3.75 - 5; n=5). Animals were dried off approximately 7 weeks before the expected parturition date. Cows were housed at the farm and one month before the expected parturition date, animals were transported to the Faculty of Veterinary Medicine (Ghent University, Belgium) to allow for environmental adaptation. During the dry period, animals were fed rations based on corn silage and grass hay or straw (Table 1). At the farm, cows were offered the lactation ration diluted with chopped straw (ratio 67:33 on a DM basis) to allow for ad libitum intake without overfeeding. At the faculty, cows were offered grass hay for ad libitum intake and 6 kg DM of corn silage each day. From two months before the expected parturition date until the end of the study, cows were weekly monitored for BCS and backfat thickness (BFT) (Schröder and Staufenbiel, 2006) by the same person and blood samples were taken from the coccygeal vein. Serum samples of the last 2 weeks before the HEC test were analyzed for NEFA concentration.

3.2. Hyperinsulinemic euglycemic clamp test

Hyperinsulinemic euglycemic clamp tests were performed in the third week (16 to 20 days) before expected parturition date as described by Kusenda et al. (2013). The day before the HEC test, cows were weighed and catheters (Cavafix Certo 338 - 14G, B.Braun, Instrulife, Oostkamp, Belgium) were fitted into both jugular veins. Water and hay were always available and corn silage was withheld from the cows from 12 hours before the HEC test until the end of the HEC test.

Blood samples were obtained from the right jugular catheter. Three blood samples were collected with 10 minutes interval to determine basal glucose, NEFA and insulin concentrations. Following assessment of basal blood glucose concentration, 4 consecutive insulin infusions were administered through the left jugular catheter at increasing doses of insulin: 0.1; 0.5; 2; and 5 mIU/kg/min (Actrapid 100 IU/mL, human recombinant insulin, Novo Nordisk, diluted in 0.9% NaCl, B.Braun, Nerum, Heusden-Zolder, Belgium).

Table 1. Ingredients and chemical composition of the rations at the farm and at the faculty

	At the farm	At the faculty
Ingredient (% , DM basis)		
Corn silage	25.42	50
Grass silage	9.46	-
Grass hay	2.53	50
Soybean meal	1.52	-
Canola meal	6.17	-
Corn meal	2.62	-
Wheat	1.35	-
Malt distillers grain	3.54	-
Beet pulp	7.35	-
Corn gluten feed	6.59	-
Rape straw	33.45	-
Chemical composition		
DM (%)	43	43.5
NE _l (Mcal/kg) ¹	1.29	1.44
CP (%)	12	11.3
NDF (%)	50.7	53.1
ADF (%)	29.8	30.2
ADL (%)	2.6	9.5
Starch (%)	13.0	20.0
Crude fat (%)	2.0	3.25
Ash (%)	4.3	6.35

¹ Net energy for lactation as estimated by NRC (2001).

The speed of each insulin infusion was set at a constant rate of 1 mL/min for 140 minutes using an infusion pump (REGLO Digital MS-2/6, Ismatec, Voor't Labo, Eeklo, Belgium). Potassium chloride (KCl, P9541, Sigma-Aldrich, Bornem, Belgium) was administered to the animals during the insulin infusions of 2 and 5 mIU/kg/min at a speed of 1 mmol/min to avoid hypokalemia. At regular time intervals (2.5 or 5 minutes) the blood glucose concentration was determined using a glucometer (Precision Xceed, Abbott Diabetes Care, Verdifarm, Beringen-Paal, Belgium). Based on the measured blood glucose concentration, the speed of a concomitant glucose infusion (30% glucose, Eurovet, Verdifarm, Beringen-Paal, Belgium, through the left jugular catheter) was adapted to keep the blood glucose concentration near basal levels. At 90; 100; 110; 120; 130; and 140 minutes after the start of each insulin infusion, blood samples were taken for determination of NEFA and insulin. After the end of the last insulin infusion, the glucose infusion was continued until the animal required no exogenous glucose to maintain a normal blood glucose concentration.

The steady state (SS) was defined as a period of 30 minutes during the last 50 minutes of the infusion period where no or minor changes of the glucose infusion were necessary to keep the blood glucose concentration constant and near basal levels. Therefore, the CV of the glucose infusion rate (GIR) and blood glucose needed to be less than 10%. During each SS, the steady state glucose infusion rate (SSGIR), the steady state insulin concentration (SSIC) and the steady state NEFA concentration (SSNEFA) were calculated as the average GIR, the average insulin concentration and the average NEFA concentration during the SS period, respectively.

3.3. Blood analyses

Blood glucose concentrations were determined using a glucometer (Precision Xceed), the intra-assay CV was 7.8%. Samples for insulin and NEFA determination were taken in gel-coated blood tubes (Vacutest, Novolab, Geraardsbergen, Belgium) centrifuged for 20 minutes (2,460 x g, 7°C) within 2 hours and stored at -80°C until analysis. Serum insulin concentrations were determined at the Clinical Chemistry Lab of the University Hospital (Ghent, Belgium) using a human specific insulin electrochemiluminiscent immunoassay (ECLIA - Roche), intra- and inter-assay CV were respectively 1.1% and 6.0%. Serum NEFA concentrations were determined in a commercial lab (Mediclab, Aalst, Belgium) using an enzymatic endpoint method, intra- and inter-assay CV were respectively 1.0% and 1.1%.

3.4. Euthanasia and adipose tissue dissection

One week after the HEC test (10 to 13 days before expected parturition date), cows were euthanized at the Department of Morphology (Faculty of Veterinary Medicine, Ghent University, Belgium). The day before euthanasia, live body weight of the cows was determined using a large animal floor scale (Bascules Robbe NV, Torhout, Belgium). Cows were stunned with a captive bolt gun and exsanguinated by transecting the carotid arteries and the jugular veins. Immediately after euthanasia, adipose tissue was dissected, weighed and subdivided in 4 depots: the subcutaneous, the abdominal, the intrapelvic, and the thoracal adipose depot. The subcutaneous adipose depot contains all the adipose tissue that is located subcutaneously at the back, the sternum, and at the tailbase (including the adipose tissue in the fossa ischiorectalis). The abdominal adipose depot contains all the adipose tissue that is located in the omentum majus, omentum minus, mesenterium, and the perirenal and retroperitoneal adipose tissue. The

intrapelvic adipose depot contains all the adipose tissue that is located in the pelvic cavity. The thoracic adipose depot contains all the adipose tissue that is located at the inside of the ribs, the adipose tissue in the mediastinum, and the adipose tissue around the heart (including the adipose tissue in the coronary grooves).

3.5. Statistical analyses

All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Descriptive statistics are expressed as mean \pm SEM unless otherwise indicated. Normality of the data and error terms of the models were checked using the Kolmogorov-Smirnov test ($P < 0.01$). One cow was excluded from the statistical analysis because steady state conditions were not reached in 2 of the 4 insulin infusion periods of the HEC test. Significance and tendency were declared at $P < 0.05$ and $0.05 < P < 0.1$ respectively.

3.5.1. BCS and BFT during the dry period

BCS and BFT during the dry period were modeled using the PROC MIXED function with cow as random factor and time points during the dry period as repeated measurements within cow. Significant differences between time points were checked using the Bonferroni adjustment for multiple comparisons.

3.5.2. Dose response curves of the glucose metabolism

During the HEC test, the SSGIR reflects the insulin response of the glucose metabolism. Because this response is measured at increasing insulin concentrations, the HEC test gives the possibility to create dose response curves between the SSGIR and the SSIC.

For each individual animal, the SSGIR was plotted with the SSIC and dose response curves were fitted using the PROC NLIN function using the following equation (Motulsky and Christopoulos, 2004):

$$SSGIR = \frac{max_{glucose}}{1 + 10^{(logEC50_{glucose} - logSSIC)}}.$$

The shape of the dose response curve is determined by 2 parameters: $max_{glucose}$ being the maximal SSGIR at maximal SSIC and $EC50_{glucose}$ being the insulin concentration needed to achieve the half maximal effect.

3.5.3. *Dose response curves of the fatty acid metabolism*

During the HEC test, the SSNEFA concentration reflects the insulin response of the fatty acid metabolism. Because the basal NEFA concentration was different between animals, SSNEFA concentrations were expressed as a percentage of the basal NEFA concentration (%NEFA), to be able to compare the response of the fatty acid metabolism between different animals. The NEFA response is measured at increasing insulin concentrations during the HEC test, giving the possibility to create dose response curves between the %NEFA and the SSIC. For each individual animal, the %NEFA was plotted with the SSIC and dose response curves were fitted using the PROC NLIN function using the following equation (Motulsky and Christopoulos, 2004):

$$\%NEFA = min_{NEFA} + \frac{1 - min_{NEFA}}{1 + 10^{(logSSIC - logEC50_{NEFA})}}$$

The shape of the dose response curve is determined by 2 parameters: min_{NEFA} being the minimal %NEFA at maximal SSIC and $EC50_{NEFA}$ being the insulin concentration needed to achieve the half maximal effect.

3.5.4. *Influence of body fatness on the parameters derived from the dose response curves.*

The parameters derived from the individual dose response curves ($max_{glucose}$, $EC50_{glucose}$, min_{NEFA} , $EC50_{NEFA}$) were used to explore the influence of body fatness on the insulin response of the glucose and fatty acid metabolism. For use in the statistical models, average values of BCS, BFT, and NEFA during the dry period within individual animals were calculated. Because the different variables reflecting body fatness were highly correlated ($r > 0.60$), univariate regression models were built to avoid multicollinearity. Influences on the parameters of the glucose and fatty acid metabolism were analyzed using a univariate regression model with $max_{glucose}$, $EC50_{glucose}$, min_{NEFA} , $EC50_{NEFA}$ as dependent variables; BCS, BFT, NEFA level during the dry period, and the absolute and proportional amount of the different adipose depots as independent variables.

3.5.5. *Comparison of EC50 between glucose and fatty acid metabolism.*

The EC50 of the glucose and fatty acid metabolism reflects the insulin concentration needed to achieve the half maximal effect of the glucose or fatty acid metabolism. Because both values were determined within the same individual, it is possible to compare the EC50 of the glucose

and the EC50 of the fatty acid metabolism within individual cows. Therefore, a mixed linear model was created with EC50 as dependent variable, metabolic path (glucose or fatty acid metabolism) as independent variable and cow as random factor.

4. RESULTS

4.1. Animal characteristics

Animal characteristics during the dry period are given in Table 2. The average BCS of the experimental animals during the dry period was 3.85 (SD = 0.67). Five animals were overconditioned (BCS > 3.75) while four animals had a normal BCS (BCS < 3.75). The average BFT of the experimental animals during the dry period was 2.39 cm (SD = 1.15). The average dry period NEFA concentration in the two weeks preceding the HEC test was 0.27 mmol/L (SD = 0.18). Cows with a high NEFA level had higher BCS ($r = 0.79$; $P < 0.05$) and higher BFT ($r = 0.83$; $P < 0.01$). BCS and BFT were not correlated with the basal insulin concentration at the start of the HEC test ($r = 0.33$ and 0.17 ; $P = 0.38$ and 0.66 for BCS and BFT respectively).

Table 2. Animal characteristics during the dry period

Item	mean	SD	min	max
BCS ¹	3.85	0.67	3.11	5.00
BFT ² (cm)	2.39	1.15	1.20	4.58
NEFA ³ (mmol/L)	0.27	0.18	0.08	0.55

¹ Body condition score, individual BCS was calculated as the average BCS during the dry period.

² Backfat thickness, individual BFT was calculated as the average BFT during the dry period.

³ Serum non esterified fatty acid concentration, individual NEFA was calculated as the average serum NEFA concentration in the 2 weeks preceding the hyperinsulinemic euglycemic clamp test.

4.2. Adipose depot weight

The body weight of the cows the day before euthanasia ranged from 631 kg to 858 kg. The weight of total body fat, the weight of the different fat depots and proportional distribution of body fat in different adipose depots are given in Table 3. The thinnest cow had only 29.62 kg of total body fat while the fattest cow had 114.26 kg of total body fat, indicating a large variation in total body fat and weight of the adipose depots. However, the proportional distribution of adipose tissue among the different depots showed very little variation. The largest amount of body fat was localized in the abdominal cavity (66.70 % of the total body fat; SD = 3.38), followed by the subcutaneous adipose tissue (17.99 %; SD = 2.19), the adipose tissue in the thoracic cavity (9.04 %; SD = 1.75), and in the pelvic cavity (6.27 %; SD = 1.17).

Table 3. Body weight, weight of total body fat and weight of the different adipose depots and the proportion of the weight of the different adipose depots

Item	Subset	mean	SD	min	max
Body weight (kg) ¹		724	78	631	858
Total body fat (kg) ²		58.01	31.24	29.62	114.26
Subcutaneous fat ³	Weight (kg)	10.55	6.45	4.76	25.56
	Proportion of total body fat (%)	17.99	2.19	14.76	22.37
Abdominal fat ⁴	Weight (kg)	38.98	21.47	19.88	73.36
	Proportion of total body fat (%)	66.70	3.38	62.09	73.24
Intrapelvic fat ⁵	Weight (kg)	3.47	1.69	1.74	7.38
	Proportion of total body fat (%)	6.27	1.17	4.60	8.03
Thoracal fat ⁶	Weight (kg)	5.01	2.49	3.08	10.36
	Proportion of total body fat (%)	9.04	1.75	5.86	11.08

¹ Body weight determined the day before euthanasia.

² Total body fat is the sum of the abdominal, subcutaneous, intrapelvic and thoracal fat.

³ Subcutaneous fat contains all the dissected fat located subcutaneously at the back, the sternum and the tailbase (including the fat in the fossa ischioirectalis).

⁴ Abdominal fat contains all the dissected fat located in the omentum majus, omentum minus, mesenterium and the perirenal and retroperitoneal fat.

⁵ Intrapelvic fat contains all the dissected fat located in the pelvic cavity.

⁶ Thoracal fat contains all the dissected fat located at the inner side of the ribs, the mediastinum and around the heart.

4.3. Hyperinsulinemic euglycemic clamp test

Figure 1 depicts the changes in blood glucose, insulin and NEFA concentrations and GIR during the HEC tests. Infusion of human insulin increased the circulating insulin concentration and decreased circulating NEFA concentrations. To maintain blood glucose values near basal concentrations, the concomitant glucose infusion was increased to reach a SS between 90 and 140 minutes after the start of the insulin infusion (Table 4).

Table 4. SSIC¹, SSGIR², SSNEFA³ and %NEFA⁴ during the basal state and the different insulin infusion periods⁵.

Insulin infusion	SSIC (μ IU/mL)	SSGIR (μ mol/kg/min)	SSNEFA (mmol/L)	%NEFA
basal state	1.09 \pm 0.08	0	0.83 \pm 0.05	100
0.1 mIU/kg/min	8.52 \pm 0.33	1.44 \pm 0.05	0.58 \pm 0.02	73.05 \pm 1.73
0.5 mIU/kg/min	51.02 \pm 1.73	8.77 \pm 0.28	0.24 \pm 0.01	29.50 \pm 1.41
2 mIU/kg/min	337.24 \pm 12.70	13.61 \pm 0.32	0.13 \pm 0.01	15.04 \pm 0.88
5 mIU/kg/min	1,432.86 \pm 52.46	18.33 \pm 0.45	0.10 \pm 0.01	12.07 \pm 0.57

¹ Steady state insulin concentration.

² Steady state glucose infusion rate.

³ Steady state NEFA concentration.

⁴ %NEFA calculated as % of the basal NEFA value.

⁵ Values are given as mean \pm SEM.

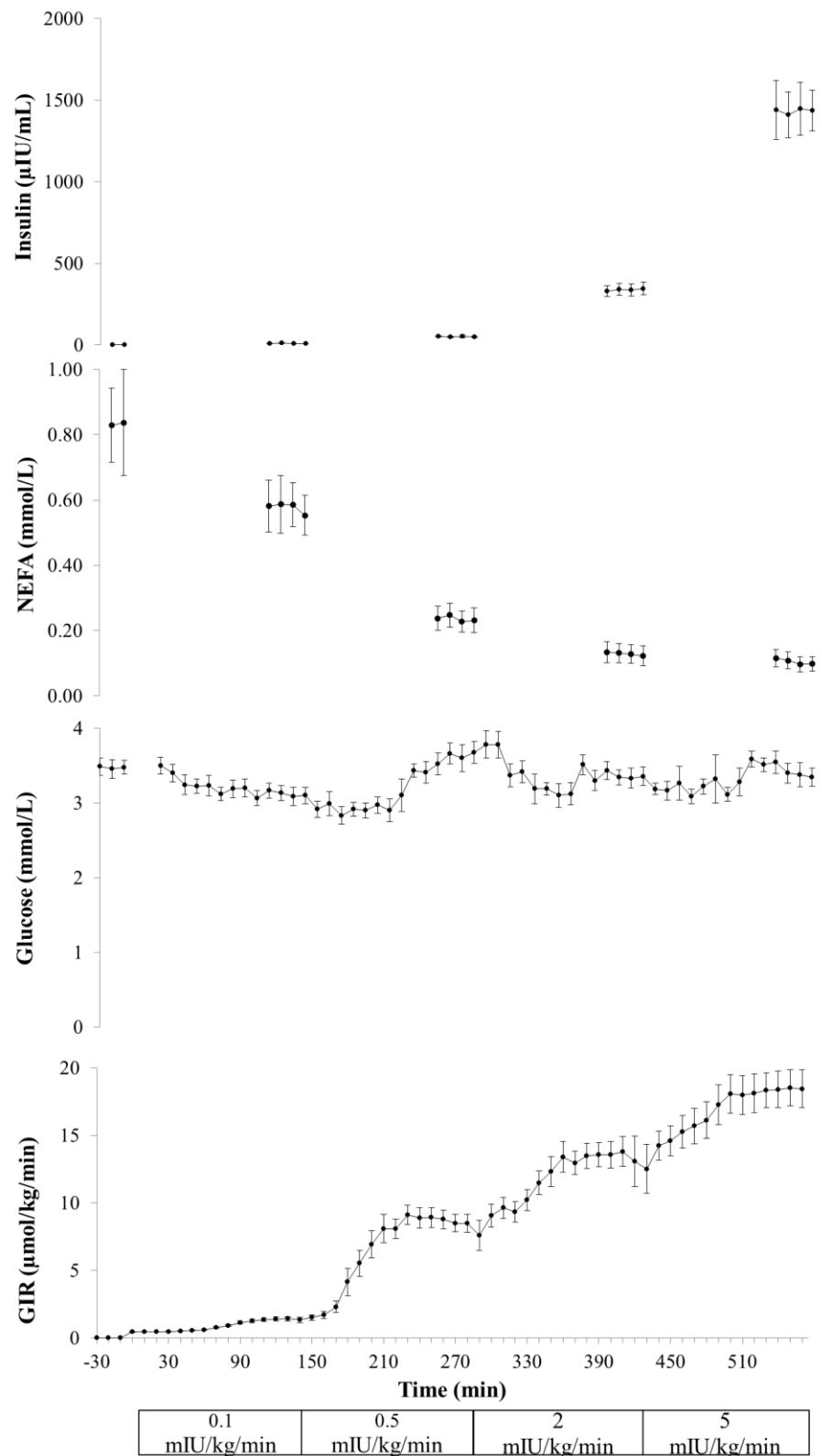


Figure 1. Insulin, NEFA and glucose concentration and glucose infusion rate (GIR) in the basal state and during the insulin infusion periods (0.1, 0.5, 2 and 5 mIU/kg/min) of the hyperinsulinemic euglycemic clamp test. Points (•) represent the means and error bars represent the standard errors of the mean (n = 9).

4.4. Parameters derived from the dose response curves of the glucose and fatty acid metabolism

Figure 2 depicts the average values of the SSGIR and SSIC of the experimental animals together with the fitted dose response curves. The dose response curve of the insulin response of the glucose metabolism is determined by the $EC50_{\text{glucose}}$ and the max_{glucose} . In the present experiment, the average values of the $EC50_{\text{glucose}}$ and max_{glucose} were respectively 76.41 ± 4.46 $\mu\text{IU/mL}$ and 18.51 ± 0.42 $\mu\text{mol/kg/min}$ (Table 5). The max_{glucose} was negatively associated with BCS, BFT, average NEFA concentration in the 2 weeks preceding the HEC test, and the absolute weight of the different adipose depots (Table 6, Figure 4). The $EC50_{\text{glucose}}$ was positively associated with BCS, BFT, average NEFA concentration in the 2 weeks preceding the HEC test, and the absolute weight of the different adipose depots. The proportional distribution of the different adipose depots was not related to the $EC50_{\text{glucose}}$ and the max_{glucose} (Table 6, Figure 4).

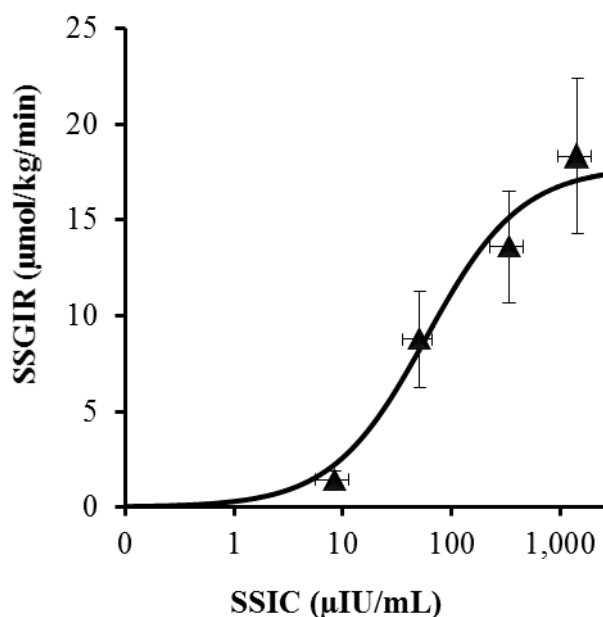


Figure 2. Data points of the experimental animals for the steady state insulin concentration (SSIC) and the steady state glucose infusion rate (SSGIR) during the hyperinsulinemic euglycemic clamp test. Points (\blacktriangle) represent the means, horizontal error bars represent the standard error of the mean SSIC and vertical error bars represent the standard error of the mean SSGIR. The line represents the fitted dose response curve from the nonlinear equation $SSGIR = \frac{max_{\text{glucose}}}{1 + 10^{(\log EC50_{\text{glucose}} - \log SSIC)}}$ with max_{glucose} being the maximal SSGIR at maximal SSIC and the effective concentration 50 ($EC50_{\text{glucose}}$) being the insulin concentration needed to achieve half maximal effect.

Table 5. Model parameters of the dose response curves of the insulin response of the glucose and fatty acid metabolism

Item	Parameter	Mean \pm SEM
Glucose metabolism ¹	max _{glucose} ($\mu\text{mol/kg/min}$)	18.51 \pm 0.42
	EC50 _{glucose} ($\mu\text{IU/mL}$)	76.41 \pm 4.46
Fatty acid metabolism ²	min _{NEFA} (%)	9.87 \pm 0.66
	EC50 _{NEFA} ($\mu\text{IU/mL}$)	19.35 \pm 1.48

¹ Model parameters of the dose response curve of the glucose metabolism were fitted using the following equation:
$$= \frac{\text{max}_{\text{glucose}}}{1 + 10^{(\log \text{EC50}_{\text{glucose}} - \log \text{SSIC})}}$$
. The shape of the dose response curve is determined by: max_{glucose} being the maximal steady state glucose infusion rate (SSGIR) at maximal steady state insulin concentration (SSIC) and EC50_{glucose} being the insulin concentration needed to achieve half maximal effect.

² Model parameters of the dose response curve of the fatty acid metabolism were fitted using the following equation:
$$\% \text{NEFA} = \text{min}_{\text{NEFA}} + \frac{1 - \text{min}_{\text{NEFA}}}{1 + 10^{(\log \text{SSIC} - \log \text{EC50}_{\text{NEFA}})}}$$
. The shape of the dose response curve is determined by: min_{NEFA} being the minimal %NEFA at maximal SSIC and EC50_{NEFA} being the insulin concentration needed to achieve half maximal effect.

Figure 3 depicts the average values of the %NEFA and the SSIC together with the fitted dose response curve. The dose response curve of the insulin response of the fatty acid metabolism is determined by the EC50_{NEFA} and the min_{NEFA}. In the present experiment, the average values of the EC50_{NEFA} and min_{NEFA} were respectively 19.35 \pm 1.48 $\mu\text{IU/mL}$ and 9.87 \pm 0.66 % (Table 5). The decrease in NEFA relative to the basal NEFA concentration (min_{NEFA}) was positively associated with the average NEFA concentration during the 2 weeks preceding the HEC test (Table 6, Figure 4). The BCS, BFT, and absolute and proportional amount of the different adipose depots were not significantly associated with the EC50_{NEFA} and the min_{NEFA} of the fatty acid metabolism.

The mixed linear model revealed that the EC50_{glucose} of the insulin response of the glucose metabolism was significantly larger than the EC50_{NEFA} of the insulin response of the fatty acid metabolism ($P < 0.001$).

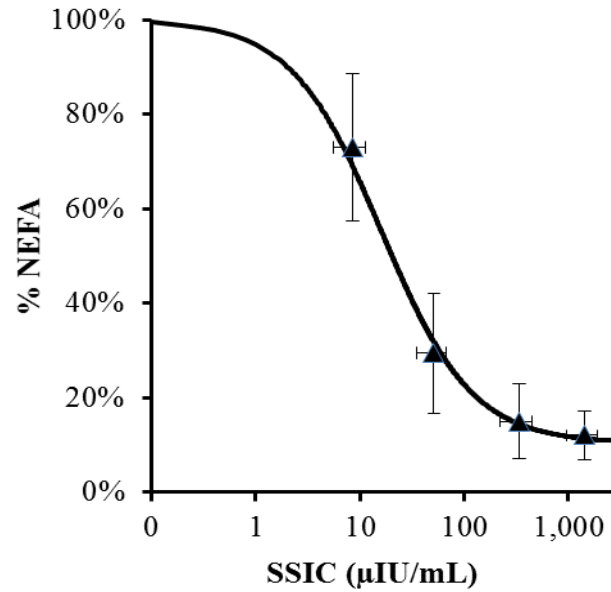


Figure 3. Data points of the experimental animals for the steady state insulin concentration (SSIC) and the procentual NEFA level compared to basal (%NEFA) during the hyperinsulinemic euglycemic clamp test. Points (▲) represent the means, horizontal error bars represent the standard error of the mean SSIC and vertical error bars represent the standard error of the mean %NEFA. The line represents the fitted dose response curve from the nonlinear equation $\%NEFA = min_{NEFA} + \frac{1-min_{NEFA}}{1+10^{(logSSIC-logEC50_{NEFA})}}$ with min_{NEFA} being the minimal SSNEFA at maximal SSIC and the effective concentration 50 ($EC50_{NEFA}$) being the insulin concentration needed to achieve half maximal effect.

Table 6. Estimates ($\beta \pm$ standard error) of the univariate regression models describing the relationship between animal characteristics and depot weights with the parameters of the insulin dose response curves of the glucose and fatty acid metabolism

Item	Glucose metabolism		Fatty acid metabolism	
	$max_{glucose}$ ($\mu\text{mol/kg/min}$)	$EC50_{glucose}$ ($\mu\text{IU/mL}$)	min_{NEFA} (%)	$EC50_{NEFA}$ ($\mu\text{IU/mL}$)
BCS	- 4.99 \pm 1.00 **	25.36 \pm 5.63 **	0.047 \pm 0.028	- 3.51 \pm 7.36
BFT ¹ (cm)	- 2.52 \pm 0.81 *	14.16 \pm 3.75 **	0.025 \pm 0.017	- 4.20 \pm 4.09
NEFA ² (mmol/L)	- 14.77 \pm 5.92 *	93.42 \pm 25.39 *	0.27 \pm 0.08 *	- 19.97 \pm 27.53
Total body fat (kg)	- 0.10 \pm 0.03 **	0.53 \pm 0.13 **	0.00090 \pm 0.00063	- 0.16 \pm 0.15
Subcutaneous fat (kg)	- 0.49 \pm 0.12 **	2.43 \pm 0.72 *	0.0038 \pm 0.0032	- 0.58 \pm 0.74
Abdominal fat (kg)	- 0.14 \pm 0.04 **	0.77 \pm 0.20 **	0.0014 \pm 0.0009	- 0.25 \pm 0.21
Pelvic fat (kg)	- 1.93 \pm 0.44 **	9.22 \pm 2.85 *	0.015 \pm 0.012	- 1.91 \pm 2.89
Thoracal fat (kg)	- 1.15 \pm 0.38 *	5.52 \pm 2.24 *	0.0068 \pm 0.0087	- 1.82 \pm 1.90
Abdominal fat ³ (%)	- 11.37 \pm 42.23	180.89 \pm 223.46	0.79 \pm 0.59	- 145.84 \pm 138.42
Subcutaneous fat ³ (%)	- 46.48 \pm 63.16	179.64 \pm 358.74	- 0.16 \pm 1.03	145.66 \pm 223.37
Pelvic fat ³ (%)	83.52 \pm 118.29	- 663.67 \pm 626.88	- 1.38 \pm 1.85	479.34 \pm 389.58
Thoracal fat ³ (%)	78.08 \pm 76.72	- 662.50 \pm 367.43	- 2.13 \pm 1.01	101.64 \pm 285.92

¹ Backfat thickness.

² Serum non esterified fatty acid concentration.

³ Depot weight calculated as percentage of total body fat.

* $P < 0.05$, ** $P < 0.01$

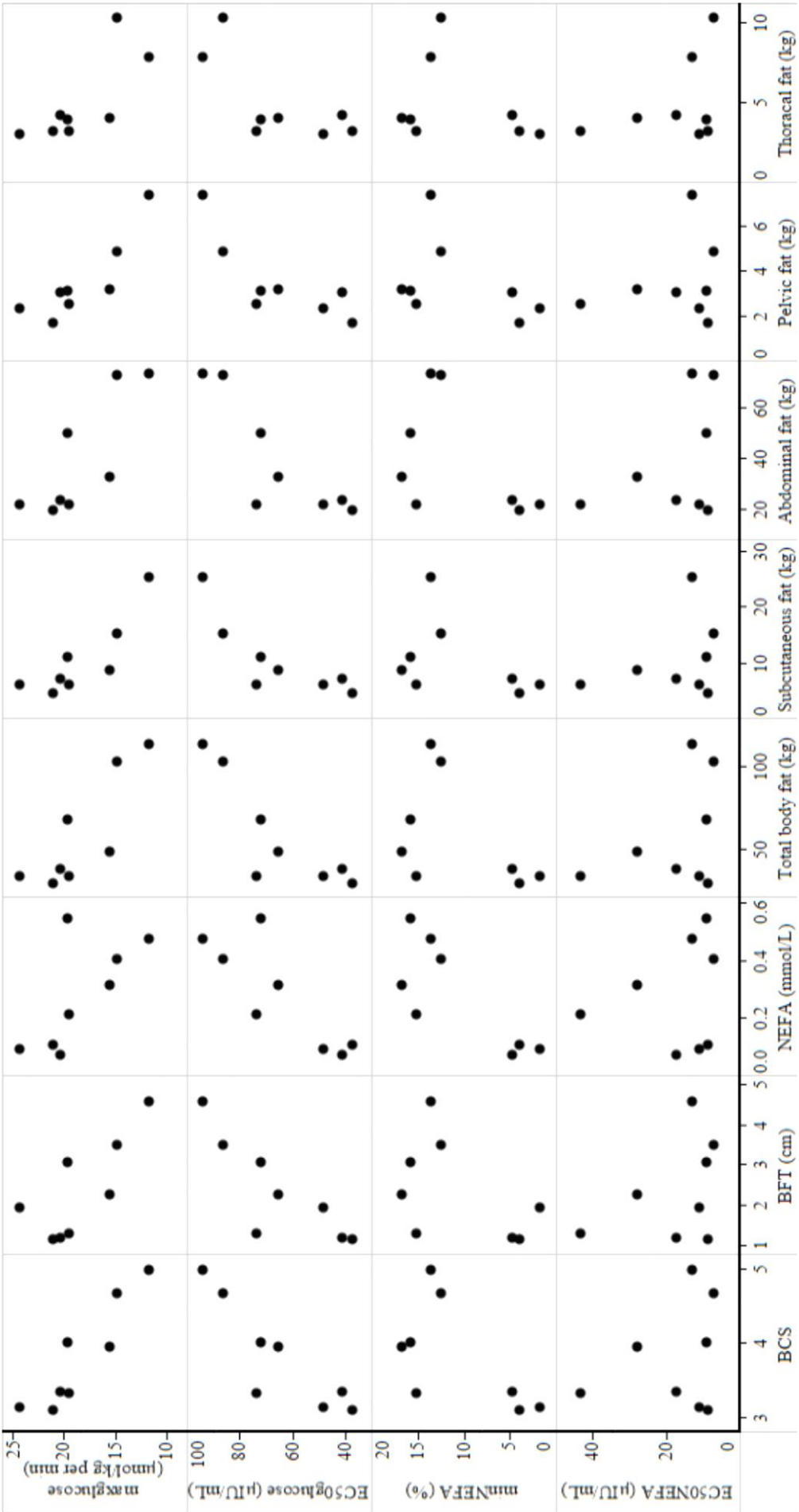


Figure 4. Visualization of the relationship between the parameters derived from the dose response curves of the glucose and fatty acid metabolism and the different variables representing the level of fat accumulation in the experimental animals. Points (•) represent the observations of the individual animals. Max_{glucose} and min_{NEFA} represent the maximal insulin response of the glucose and NEFA metabolism, respectively. EC50_{glucose} and EC50_{NEFA} represent the insulin concentration needed to achieve the half maximal effect of the glucose and NEFA metabolism, respectively.

5. DISCUSSION

The aim of the present study was to determine the influence of the level of fat accumulation on the insulin response of the glucose and fatty acid metabolism in dairy cows. We hypothesized that the insulin response of overconditioned cows may be attenuated. Because overconditioning of dairy cows is mainly a problem in dry dairy cows that can negatively impact early lactation performance (Roche et al., 2013), experiments were conducted on dairy cows at the end of the dry period. Animals in the present study were fed according to their requirements to avoid the influence of over- and underfeeding on the insulin response of the glucose and fatty acid metabolism as has been demonstrated by Holtenius et al. (2003) and Schoenberg et al. (2012). In the present study, the comparison of BCS and BFT at different time points within individuals, revealed no significant difference, meaning that the BCS and BFT did not change during the dry period and supporting the fact that no animals were significantly under- or overfed.

Five overconditioned and four normal conditioned animals successfully completed this study in which total body insulin response of the glucose and fatty acid metabolism was determined using the HEC test. The HEC test is the gold standard method to determine the insulin response in humans and animals (DeFronzo et al., 1979). The HEC test allows to determine the total body glucose uptake at supraphysiological concentrations of insulin, thereby giving the possibility to create dose response curves and to determine the insulin concentration needed to elicit half maximal effect (EC50) and the maximal effect of insulin.

5.1. Insulin response of the glucose metabolism

During the HEC test, the SSGIR reflects the insulin-mediated increase in total body glucose metabolism and is the resultant of both the decrease in endogenous glucose production and the increase in glucose uptake by skeletal muscle and adipose tissue (DeFronzo et al., 1979). Skeletal muscle is the most important contributor to this glucose uptake. As such, the insulin response of the glucose metabolism during the HEC test reflects mainly the insulin response of skeletal muscle (DeFronzo and Tripathy, 2009). The most important finding of the present study is that the insulin response of the glucose metabolism in dry dairy cows is negatively associated with excessive accumulation of adipose tissue. This impaired insulin action in

overconditioned dairy cows is characterized by an increased $EC_{50_{\text{glucose}}}$ and a decreased maximal effect of insulin (max_{glucose}) and can hence be described as respectively decreased insulin sensitivity and decreased insulin responsiveness (Kahn, 1978).

Impaired insulin action in obese sheep and obese heifers has been demonstrated (McCann and Reimers, 1985; Bergman et al., 1989), however this impaired insulin action was only characterized by decreased insulin sensitivity while the insulin responsiveness remained unchanged. The difference with our findings may be explained by differences in the experimental setup and by the fact that in our study, animals were in the last month of gestation being a period in which homeorhetic adaptations may modulate the insulin response of the glucose metabolism (Bell and Bauman, 1997).

The obtained values for $EC_{50_{\text{glucose}}}$ and maximum SSGIR (max_{glucose}) of the glucose metabolism in the present study are comparable with experiments performed in sheep (Janes et al., 1985; Bergman et al., 1989; Petterson et al., 1993). As expected, the maximum SSGIR was lower compared to lactating cows (Kusenda et al., 2013). The difference between the maximum SSGIR in dry and lactating cows can be explained by the high insulin independent glucose uptake of the lactating mammary gland. Indeed, glucose uptake by the mammary gland at 14 days in milk increases total glucose requirements when compared to the dry period (De Koster and Opsomer, 2013).

5.2. Insulin response of the fatty acid metabolism

During the HEC test, the decrease of the SSNEFA compared to basal NEFA level reflects the inhibitory effect of insulin on the circulating NEFA. This is achieved by the combined effect of insulin to inhibit lipolysis and to stimulate lipogenesis (Campbell et al., 1992; Frayn et al., 1997). Obtained values for the $EC_{50_{\text{NEFA}}}$ and minimal %NEFA (min_{NEFA}) of the fatty acid metabolism are comparable with similar experiments performed in sheep (Petterson et al., 1994) and lactating dairy cows (Kusenda et al., 2013). The insulin action at the level of the fatty acid metabolism was not associated with the amount of accumulated adipose tissue. Similarly, overfeeding dairy cows during the dry period did not impair insulin signaling in subcutaneous adipocytes (Ji et al., 2012).

Differences in the regulatory mechanisms of insulin between the glucose and fatty acid metabolism are known to occur in humans (Arner et al., 1984) and have recently been demonstrated in dry dairy cows as well (Schoenberg et al., 2012). The present findings suggest specific pathways by which excessive accumulation of fat impairs insulin response of the glucose metabolism without altering insulin response of the fatty acid metabolism in dry dairy cows. It has been reported that skeletal muscle insulin resistance may develop in the absence of insulin resistance of the adipose tissue (Crettaz and Jeanrenaud, 1980; Kahn and Flier, 2000). In the present study, only the insulin response of the glucose metabolism is decreased, reflecting reduced insulin response of the glucose uptake by skeletal muscle. A drawback of the difference in insulin response between the glucose and fatty acid metabolism is that results obtained based on the glucose metabolism, may not be extrapolated as such to the fatty acid metabolism.

5.3. Comparison between the insulin response of the glucose and fatty acid metabolism

Comparison of EC₅₀ between the glucose and fatty acid metabolism revealed lower insulin sensitivity of the glucose metabolism compared to the fatty acid metabolism in the same individual which is in agreement with observations in pregnant sheep (Pettersson et al., 1993; Pettersson et al., 1994). This observation implies that the antilipolytic effect of insulin occurs at much lower insulin concentrations compared to the stimulatory effect of insulin on the glucose transport (Kahn and Flier, 2000). Similarly, Vernon (2005) described different levels of insulin sensitivity for different metabolic processes; anabolic processes (glycogen, protein, and triacylglycerol synthesis) and peripheral glucose uptake are less sensitive to insulin compared to catabolic processes like lipolysis and proteolysis. In the perspective of treating cows which mobilize excessive amounts of NEFA in the periparturient period, this distinction between the glucose and fatty acid metabolism may be favorable. Because treatment is based on inhibition of lipolysis by increasing circulating concentrations of glucose and insulin, and the action of insulin at the level of the fatty acid metabolism remains intact in overconditioned cows. Additionally, the half maximal effect of insulin on the fatty acid metabolism is achieved at low levels of insulin which are easily reached by intravenous infusion of a glucose solution

(Holtenius et al., 2003) and peroral bolus administration of glucogenic precursors (Nielsen and Ingvarsten, 2004). Present observations support current treatment protocols although care has to be taken when extrapolating observations from dry to lactating cows.

5.4. Role of NEFA and insulin in the insulin response of the glucose and fatty acid metabolism

Impairment of insulin's action associated with obesity can be explained by hyperinsulinemia, increased concentration of circulating NEFA and altered production of adipokines. Hyperinsulinemia causes downregulation of the number of insulin receptors in adipose tissue, skeletal muscle and liver thereby inducing a state of decreased insulin sensitivity (Crettaz and Jeanrenaud, 1980; DeFronzo and Tripathy, 2009). Overfeeding dairy cows during the dry period is associated with hyperinsulinemia and insulin resistance (Holtenius et al., 2003). However, in the present research, overconditioning was not associated with a state of hyperinsulinemia as indicated by the non-significant correlation between basal insulin concentration at the start of the HEC test and BFT and BCS. Serum insulin levels start to decline in the last month before parturition (Accorsi et al., 2005; Kokkonen et al., 2005), due to the increased glucose requirements of the gravid uterus and the developing mammary gland (Bauman and Currie, 1980; Bell et al., 1995). As such, the stimuli to the pancreas to secrete insulin are decreased, when cows are fed according to their requirements. Probably, homeorhetic mechanisms in periparturient dairy cows overrule the effect of obesity on hyperinsulinemia and different mechanisms may be responsible for the development of insulin resistance between overconditioned and overfed cows.

As expected, cows with high BCS and BFT had also higher concentrations of circulating NEFA during the weeks preceding HEC tests, indicating precalving mobilization of body fat (Grummer, 1993; Kokkonen et al., 2005). High levels of circulating NEFA are known to negatively impact the effect of insulin at the level of the glucose and fatty acid metabolism as demonstrated experimentally in dairy cows by intravenous infusion of tallow emulsion (Pires et al., 2007; Salin et al., 2012) and by prolonged fasting (Oikawa and Oetzel, 2006), while in early lactation NEFA level is negatively associated with glucose decrease after insulin injection (Kerestes et al., 2009). Similarly, in the present research, impaired insulin action at the level of

the glucose metabolism ($EC_{50_{\text{glucose}}}$ and max_{glucose}) and the fatty acid metabolism (min_{NEFA}) was associated with high levels of NEFA in the weeks preceding the HEC test. Although statistically significant, it is questionable if the effect of NEFA on the minimum %NEFA is biologically relevant because estimates of the regression function are much lower compared to estimates of the regression function of the glucose metabolism. NEFA are shown to induce serine phosphorylation of IRS1, an important protein in the intracellular signaling cascade of insulin (LeMarchand-Brustel et al., 2003). Serine phosphorylation of IRS reduces insulin induced tyrosine phosphorylation of proteins thereby decreasing activation of the intracellular insulin signaling cascade (Shulman, 2000; LeMarchand-Brustel et al., 2003).

Additional factors which may influence insulin response are bioactive molecules produced by the adipose tissue, called adipokines. In human medicine, the metabolic syndrome is known as a condition which links obesity, cardiovascular disease, dyslipidemia, insulin resistance, and type II diabetes mellitus (Cornier et al., 2008). Dysregulated production of adipokines by the obese adipose tissue plays an important role in the development of insulin resistance in an endocrine, paracrine, or autocrine way. This dysregulated production is characterized by an increased production of pro inflammatory adipokines like TNF, resistin, IL6, and decreased production of adiponectin, an anti-inflammatory adipokine (Cornier et al., 2008). Recent investigations support an important role for resistin (Reverchon et al., 2014), adiponectin (Singh et al., 2014), TNF (Ohtsuka et al., 2001), and leptin (Ingvarsen and Boisclair, 2001) in the transition period of dairy cows. However, the underlying mechanism by which adipokines of enlarged adipocytes in overconditioned dairy cattle may influence insulin response of the glucose and fatty acid metabolism requires further investigation.

5.5. Role of adipose tissue distribution in the insulin response of the glucose and fatty acid metabolism

Excessive accumulation of visceral fat is an important factor in the development of insulin resistance in human medicine. The metabolic activity (release of NEFA and adipokines) and the location (direct draining to the liver) makes overaccumulation of fat in the abdominal adipose tissue more detrimental compared to subcutaneous adipose tissue (Wajchenberg, 2000). In the present study, especially the absolute amount of fat present in the body was

demonstrated to be negatively associated with the insulin response of the glucose metabolism while measures of body fat distribution (proportional distribution) were not associated with the insulin response of the glucose or fatty acid metabolism. However, limited variability existed in the distribution of body fat among the different adipose depots. Therefore it seems that an increase in BCS in the present study was associated with an increase in absolute weight of all fat depots and proportionally, all fat depots gained similar amounts of weight. Adipose tissue metabolism differs among the different adipose depots. The latter is a species specific phenomenon and within a species, some interindividual variability exists (Pond, 1992). In dairy cows, it has been demonstrated that overfeeding may increase abdominal fat depots without visible increase of subcutaneous adipose tissue (Drackley et al., 2014). While in the immediate postpartum period, there are cows with a normal amount of subcutaneous fat (normal BCS) but a considerable amount of omental fat (high omental fat score) (Hostens et al., 2012). These studies suggest variation in adipose tissue metabolism according to the location in the body of dairy cows. More research is needed to identify the potential role of the different adipose depots in the development of insulin resistance in dairy cows and the potential mechanisms by which the different adipose depots may influence health disorders in dairy cows.

6. CONCLUSIONS

The present research demonstrates a negative association between the level of fat accumulation and the insulin response of the glucose metabolism in pregnant dairy cows at the end of the dry period. This impaired insulin action at the level of the glucose metabolism is characterized by both decreased insulin sensitivity as well as decreased insulin responsiveness. Insulin response of the fatty acid metabolism on the other hand, was not associated with the level of fat accumulation. Distinction between the insulin response of the glucose and the fatty acid metabolism was also obvious for the insulin concentration needed to elicit half maximal effect. For the fatty acid metabolism, the half maximal effect was reached at lower insulin concentrations compared to the glucose metabolism.

7. ACKNOWLEDGMENTS

This research was funded by the Special Research Fund of Ghent University, grant number 01D28410. The authors wish to thank Isabel Lemahieu and Petra Van Damme for excellent technical assistance in the lab.

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CHAPTER 6

Influence of adipocyte size and adipose depot on the in vitro lipolytic activity and insulin sensitivity of adipose tissue in dairy cows at the end of the dry period

De Koster J., Van den Broeck W., Hulpio L., Claeys E., Van Eetvelde M., Hermans K., Hostens M., Fievez V. and Opsomer G. 2016. Influence of adipocyte size and adipose depot on the in vitro lipolytic activity and insulin sensitivity of adipose tissue in dairy cows at the end of the dry period. Journal of Dairy Science. 99:2319-2328.

1. ABSTRACT

The aim of the present research was to describe characteristics of *in vitro* adipose tissue lipolysis in dairy cows with a variable body condition score. Ten clinically healthy Holstein Friesian cows were selected based on BCS and were euthanized 10 to 13 days prior to the expected parturition date. Immediately after euthanasia, adipose tissue samples were collected from subcutaneous and omental fat depots. In both depots, we observed an increase in adipocyte size with increasing BCS. Using an *in vitro* explant culture of subcutaneous and omental adipose tissue, we aimed to determine the influence of adipocyte size and localization of adipose depot on the lipolytic activity in basal conditions and after addition of isoproterenol (non-selective β agonist) and insulin in different concentrations. Glycerol release in the medium was used as a measure for lipolytic activity. We observed that the basal lipolytic activity of subcutaneous and omental adipose tissue increased with adipocyte volume meaning that larger fat cells have higher basal lipolytic activity independent of the location of the adipose depot. Dose response curves were created between the concentration of isoproterenol or insulin and the amount of glycerol released. The shape of the dose response curves is determined by the concentration of isoproterenol and insulin needed to elicit the half maximal effect and the maximal amount of stimulated glycerol release or the maximal inhibitory effect of insulin. We observed that larger fat cells released more glycerol upon maximal stimulation with isoproterenol and this was more pronounced in subcutaneous adipose tissue. Additionally, larger fat cells have a higher sensitivity towards lipolytic signals. We observed a trend for larger adipocytes to be more resistant to the maximal antilipolytic effect of insulin. The insulin concentration needed to elicit the half maximal inhibitory effect of insulin was within the physiological range of insulin and was not influenced by adipocyte size or adipose depot. It can be concluded that overconditioned cows have larger adipocytes and we suggest that these overconditioned cows are predisposed to excessive mobilization of body fat due to a higher basal and stimulated lipolytic activity of large adipocytes while the antilipolytic effect of insulin is preserved.

2. INTRODUCTION

The adipose tissue is an important tissue capable of storing or mobilizing energy during periods of energy excess or deficit, respectively. During the lactation cycle of dairy cows, the adipose tissue dynamically transitions from a period of energy storage at the end of lactation through a period of energy mobilization to dampen the negative energy balance at the beginning of lactation (McNamara, 1991). At the end of the dry period and in the beginning of lactation, adipocytes go through a period of pronounced lipolysis, while *de novo* lipogenesis and re-esterification of fatty acids are down-regulated (McNamara, 1991; Vernon et al., 2001). Lipolytic pathways in adipocytes are up-regulated as part of the homeorhetic adaptation mechanism to preserve sufficient energy for the growing fetus and subsequently the lactating mammary gland. More specifically, adipocytes become insulin resistant, whereas basal and catecholamine-stimulated lipolytic activity are upregulated (Bauman and Currie, 1980; McNamara, 1991; Vernon, 2005).

Excessive mobilization of body fat during late pregnancy and early lactation may lead to elevated NEFA and BHBA levels in the blood with a negative impact on health and productivity of dairy cows (Drackley, 1999; Ospina et al., 2013). Particularly, overconditioned cows are known to be susceptible for excessive fat mobilization in the periparturient period. This is due to a more pronounced negative energy balance as a consequence of depressed appetite (Drackley, 1999; Grummer et al., 2004). Additionally, it has been hypothesized that the excessive fat mobilization in overconditioned cows may be due to the greater amount of fat available to mobilize or due to a more enhanced lipolytic activity or insulin resistance of adipocytes in adipose depots of overconditioned cows or due to a combination of these factors (Rukkwamsuk et al., 1998; Herdt, 2000; Kokkonen et al., 2005).

Furthermore, adipose depots in different regions of the body demonstrate different metabolic activities (Pond, 1992). In humans, there is ample evidence supporting a large heterogeneity in metabolic and endocrine properties between different adipose depots (Wajchenberg, 2000). Sensitivity to catecholamine-stimulated lipolysis is increased while sensitivity to the antilipolytic effect of insulin is reduced in visceral compared to subcutaneous adipocytes (Wajchenberg, 2000). As a consequence, obese individuals mobilize more NEFA from visceral

compared to subcutaneous adipose tissue leading to an overflow of NEFA in the liver with detrimental impact on health (Wajchenberg, 2000). Recent research indicates depot-specific variation in adipose tissue metabolism in dairy cows as well. Hostens et al. (2012) and Drackley et al. (2014) demonstrated different levels of fat accumulation and mobilization between different adipose depots in lactating and nonlactating dairy cows, respectively. Locher et al. (2011; 2012) demonstrated differences in metabolic activation of lipolytic enzymes between adipose depots in dry and lactating dairy cows.

The aim of the present research was to describe characteristics of adipose tissue lipolysis in dairy cows with a variable BCS at the end of the dry period. Using an *in vitro* explant culture of subcutaneous and omental adipose tissue, we aimed to determine the influence of adipocyte size and localization of adipose depot on the lipolytic activity in basal conditions and, after addition of catecholamines and insulin in different concentrations.

3. MATERIALS AND METHODS

All experimental procedures were approved by the ethical committee of the Faculty of Veterinary Medicine (EC2010/149 – Ghent University, Belgium).

3.1. Study design

Ten clinically healthy, pregnant Holstein Friesian dairy cows (upcoming parity 2 to 5) were selected at the beginning of the dry period (2 months before the expected parturition date) based on BCS according to the scale of Edmonson et al. (1989). Five animals were considered to have a normal BCS (BCS 2.5 - 3.5) and 5 animals were considered to be overconditioned (BCS 3.75 - 5). During the study, animals were fed according to their requirements. The study design is described in detail by De Koster et al. (2015).

Cows were euthanized 10 to 13 days prior to the expected parturition date at the Department of Morphology (Faculty of Veterinary Medicine, Ghent University, Belgium). Cows were stunned with a captive bolt gun and exsanguinated by transecting the carotid arteries and jugular veins. Immediately after euthanasia, adipose tissue samples were collected from subcutaneous and omental adipose tissue. Subcutaneous adipose tissue samples were taken from the adipose tissue located in the fossa ischioirectalis, while omental adipose tissue samples were taken from the omentum majus at the right side of the body.

3.2. In vitro explant culture

Immediately after sampling, adipose tissue was minced in small fragments using sharp scissors and transported to the laboratory at 38°C. Approximately 100 mg of adipose tissue fragments were transferred to a cell culture dish (35 x 10 mm, cellstar, greiner bio-one) containing 3 mL medium. The medium used in this experiment was Krebs Ringer Bicarbonate Hepes buffer (pH 7.4) containing 30 mM HEPES (H3375, Sigma Aldrich), 5.5 mM glucose (G6152, Sigma Aldrich) and 3% fatty acid free BSA (A6003, Sigma Aldrich). Culture dishes containing explants were incubated on a shaker (gentle agitation) in an incubator at 38°C. Explants were allowed to accommodate during a 20 min period. After this pre-incubation, zero time samples were taken to determine the starting value of glycerol after which hormones were added to the culture dishes. All experimental conditions were performed in duplicate. For assessment of basal lipolysis no agents were added. Solutions of isoproterenol hydrochloride (ISO, non-selective β -adrenergic receptor agonist, I6504, Sigma Aldrich) were made fresh on the day of the experiment and added to the cell culture dishes at concentrations of 10^{-6} ; 10^{-7} ; 10^{-8} and 10^{-9} M. To determine the inhibitory capacity of insulin on the lipolytic activity of the adipocytes, human insulin (I9278, Sigma Aldrich) at concentrations of 1; 10; 200 and 1,000 μ IU/mL was added simultaneously with 10^{-6} M ISO to the culture dishes. After 3 hours of incubation, samples of incubation medium were taken for determination of glycerol. Samples for determination of glycerol were stored at -20°C until analysis.

3.3. Glycerol analysis

The glycerol concentration in the culture medium was determined using free glycerol reagent (F6428, Sigma Aldrich). Samples, medium (blanco) and standards (100 μ L) were loaded in a 96 well plate and 300 μ L free glycerol reagent was added. After 10 min of incubation at room temperature, optical density was measured at 540 nm and concentration was calculated using a standard curve. Intra- and inter-assay coefficient of variation were 4.1% and 7.7%, respectively. The glycerol release was determined by subtracting the glycerol concentration in the medium after 3 hours of incubation with the zero time sample (after 20 min of pre-incubation). Results are normalized as glycerol release per million adipocytes using the number of adipocytes per gram adipose tissue (Viswanadha and Londos, 2008).

3.4. Determination of the number of adipocytes

The number of adipocytes per gram adipose tissue was determined as described by Digirolamo and Fine (2001). The weight of the explants in each culture dish was precisely determined using an analytical scale (Mettler AE 200). Diameters of the subcutaneous and omental adipocytes were determined after digestion of 1 g adipose tissue in 3 mL medium containing 2 mg/mL collagenase (C6885, Sigma Aldrich). Digestion was complete after 1 hour incubation at 38°C. Samples of the adipocyte suspension were loaded in a culture dish and the diameter of 300 adipocytes was determined using an inverted microscope (Olympus CKX41). Mean diameter (\overline{Dia}) and standard deviation of the diameter (SD_{dia}) were used to calculate the adipocyte volume ($V_{adipocyte}$) using the Goldrick formula (Goldrick, 1967):

$$V_{adipocyte} = \frac{\pi * \overline{Dia} * (3 * SD_{dia}^2 + \overline{Dia}^2)}{6}$$

The triglyceride content of the subcutaneous and omental adipose tissue was determined as described before (Lourenco et al., 2007). Briefly, between 0.5 g and 0.8 g of adipose tissue was extracted overnight with 30 mL of chloroform/methanol (2/1, vol/vol) and 3 mL of butylated hydroxytoluene in chloroform (0.1%, wt/vol). Samples were then filtered (Fiorini, S.A.) and the filtrate was collected. The filter was washed twice with 10 ml of chloroform/methanol (2/1, vol/vol). The filtrate was transferred to a flat bottom flask and evaporated with a rotary vapor (Laborota 4000 WB, Germany) at 40°C. The dry residue (103 °C during 2 hours) was cooled in a desiccator and the amount of fat was determined gravimetrically. Five-fold replicates were performed for each sample. Lipid density (ρ) was considered to be the density of triglycerides (0.915 g/mL). Number of adipocytes per gram tissue was determined using the following formula (DiGirolamo et al., 1971):

$$number\ of\ adipocytes\ per\ gram\ adipose\ tissue = \frac{triglyceride\ content}{\rho * V_{adipocyte}}.$$

3.5. Statistical analysis

All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Descriptive statistics are expressed as mean \pm SEM unless otherwise indicated. Normality of the data and error terms of the models were checked using the Kolmogorov-Smirnov test ($P < 0.01$). Interaction terms were left out the model if not

significant ($P < 0.05$). One overconditioned cow (BCS 4.0) was excluded from final statistical analysis because adipocytes failed to react on ISO. Pearson correlation coefficients were calculated to demonstrate the relationship between BCS and volume of the adipocytes, and between number of adipocytes per gram adipose tissue and volume of the adipocytes. Significance and tendency were declared at $P < 0.05$ and $0.05 < P < 0.1$, respectively.

Basal glycerol release. To check for significant effects on basal glycerol release, a model was used with basal glycerol release as dependent variable; whereas depot (subcutaneous and omental) and volume of the adipocytes were included as independent variables. A compound symmetry structure was used to model the duplicate measurements within individual animals. Because basal glycerol release was lognormally distributed, the model was fitted using a generalized linear mixed model (PROC GLIMMIX). Because BCS and volume of the adipocytes were highly correlated ($r > 0.60$), only volume of the adipocytes was included as independent variable in the model.

Isoproterenol stimulated glycerol release. The glycerol release was stimulated using 4 different ISO concentrations. Therefore, it was possible to create dose response curves for the ISO stimulated glycerol release of the adipose tissue fragments. To correct for differences in basal glycerol release between different cows and different depots, the basal glycerol release was subtracted from the ISO stimulated glycerol release, and thus the incremental glycerol release was modeled. Dose response curves were created using the PROC NLIN function using the following equation (Motulsky and Christopoulos, 2004):

$$\text{incremental glycerol release} = \frac{\max_{ISO}}{(1 + 10^{\log ED50_{ISO} - \log ISO})}$$

The shape of the dose response curve is determined by 2 parameters: \max_{ISO} being the maximal amount of glycerol released from the explants by addition of 10^{-6} M ISO and $ED50_{ISO}$ being the effective dose of ISO needed to elicit the half maximal glycerol release.

Insulin inhibited glycerol release. The maximal ISO stimulated glycerol release was inhibited using 4 different insulin concentrations (INS). Therefore it was possible to create dose response curves for the inhibitory effect of insulin on the glycerol release of the adipose tissue explants. To correct for differences in maximal ISO stimulated glycerol release, the inhibition of the glycerol release is expressed as a percentage of the maximal ISO stimulated glycerol

release without insulin. Dose response curves were created using the PROC NLIN function using the following equation (Motulsky and Christopoulos, 2004):

$$\% \text{ insulin inhibited glycerol release} = \min_{\text{INS}} + \frac{(1 - \min_{\text{INS}})}{(1 + 10^{\log \text{INS} - \log \text{ED50}_{\text{INS}}})}$$

The shape of the dose response curve is determined by 2 parameters: \min_{INS} being the minimal percentage of glycerol released from the explants (representing the maximal inhibitory effect of insulin) and ED50_{INS} being the effective dose of insulin needed to elicit the half maximal inhibitory effect of insulin. The results of one normal conditioned cow (BCS 3.0) were excluded from this part of the study because it was not possible to create dose response curves for the inhibitory effect of insulin.

Statistical analysis of parameters derived from dose response curves. For the statistical analysis of influences on the parameters of the dose response curves, different models were created with the parameters as dependent variables. Parameters \max_{ISO} and \min_{INS} were normally distributed and a mixed linear model (PROC MIXED) was used with depot and adipocyte volume as independent variables; cow was included as random factor. Parameters ED50_{ISO} and ED50_{INS} were lognormally distributed and a generalized linear mixed model (PROC GLIMMIX) was used with depot and adipocyte volume as independent variables; cow was included as random factor. Because BCS and volume of the adipocytes were highly correlated ($r > 0.60$), only volume of the adipocytes was included as independent variable in the model.

4. RESULTS

4.1. Animal and depot characteristics

The BCS of cows in the present study are given in Table 1, while other animal characteristics (backfat thickness, peripheral NEFA levels, body weight and depot weight) are presented in detail by De Koster et al. (2015). The volume of the adipocytes was comparable in both adipose depots and ranged from 0.38 nL to 1.59 nL in subcutaneous adipose tissue and from 0.23 nL to 1.62 nL in omental adipose tissue (Table 1).

Table 1. Animal and depot characteristics of subcutaneous and omental adipose tissue

Item	Mean	SD	Minimum	Maximum
BCS	3.72	0.75	2.83	5.00
Diameter of the adipocytes (μm)				
Subcutaneous	111.5	17.7	87.3	140.9
Omental	103.1	26.5	73.5	142.8
Volume of the adipocytes (nL)				
Subcutaneous	0.84	0.40	0.38	1.59
Omental	0.73	0.53	0.23	1.62
Lipid (% fat in adipose tissue)				
Subcutaneous	83.7	3.9	77.1	87.5
Omental	85.3	4.5	76.3	91.0
Number of adipocytes ($\times 10^6$ adipocytes/g adipose tissue)				
Subcutaneous	1.28	0.51	0.59	2.24
Omental	2.01	1.20	0.61	3.67

With increasing BCS, the size of the adipocytes increased linearly as demonstrated by a positive correlation between volume of the adipocytes and BCS of the animals (Figure 1; $r = 0.91$ and 0.94 in subcutaneous and omental adipose tissue, respectively, $P < 0.05$ for both). The number of adipocytes per gram adipose tissue ranged from 0.59 to 2.24×10^6 adipocytes per gram subcutaneous adipose tissue and from 0.61 to 3.67×10^6 adipocytes per gram omental adipose tissue (Table 1). Logically, the number of adipocytes per gram adipose tissue was lower in overconditioned cows compared to leaner cows, as demonstrated by the negative correlation between volume of the adipocytes and number of adipocytes per gram adipose tissue ($r = -0.93$ and -0.93 in subcutaneous and omental adipose tissue, respectively, $P < 0.05$ for both). Regarding the lipid content in both adipose depots, the collected adipose tissue fragments contained on average more than 80% of lipid indicating a low level of contamination of samples by other tissues like connective tissue and blood vessels (Table 1).

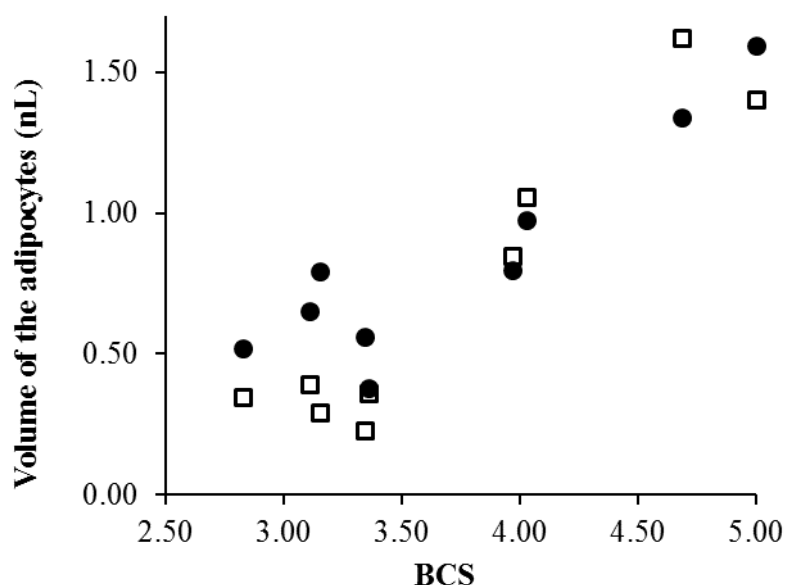


Figure 1: Visualization of the relationship between BCS of the animals and the volume of the adipocytes in subcutaneous (●) and omental (□) adipose tissue. Points represent the observations of the individual animals.

4.2. Basal glycerol release

The average basal glycerol release in subcutaneous and omental adipose tissue was 1,325 and 1,038 nmol glycerol/ 10^6 adipocytes per 3 hours, respectively (Table 2). The relationship between the volume of the adipocytes and the basal glycerol release for both depots is depicted in Figure 2. The generalized linear mixed model revealed a positive relationship between the volume of the adipocytes and the basal lipolytic activity but no significant difference could be detected between depots (Table 4).

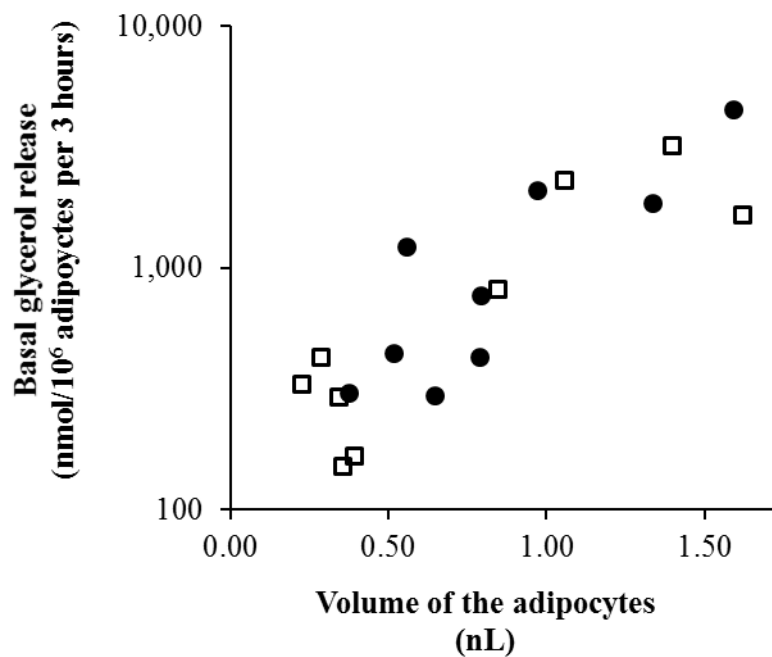


Figure 2: Visualization of the relationship between the volume of the adipocytes and the basal glycerol release in subcutaneous (●) and omental (□) adipose tissue. Points represent the observations of the individual animals.

Table 2. Glycerol release (mean \pm SEM) of subcutaneous and omental adipose tissue in the basal state, after the addition of isoproterenol (10^{-6} ; 10^{-7} ; 10^{-8} ; 10^{-9} M) or insulin (1; 10; 200; 1,000 μ IU/mL)

Item	Subcutaneous	Omental
Basal glycerol release ¹	1,325 \pm 320	1,038 \pm 258
Isoproterenol stimulated glycerol release ²		
10^{-6} M	8,935 \pm 1,027	5,748 \pm 814
10^{-7} M	7,992 \pm 990	5,896 \pm 822
10^{-8} M	7,588 \pm 1,072	4,423 \pm 746
10^{-9} M	3,035 \pm 1,002	1,310 \pm 579
Insulin inhibited glycerol release ³		
1 μ IU/mL	84.1 \pm 4.7	90.3 \pm 3.6
10 μ IU/mL	68.9 \pm 6.0	81.6 \pm 3.6
200 μ IU/mL	68.6 \pm 4.1	78.2 \pm 4.0
1000 μ IU/mL	71.4 \pm 4.9	76.7 \pm 5.3

¹ in nmol glycerol/10⁶ adipocytes per 3 hours.

² given as the incremental glycerol release above basal; in nmol glycerol/10⁶ adipocytes per 3 hours.

³ calculated as percentage of the maximal isoproterenol stimulated glycerol release without insulin; in %.

4.3. Isoproterenol stimulated glycerol release

Addition of ISO to the culture medium stimulated the glycerol release and increased the maximal glycerol release approximately 7- and 5-fold compared to the basal glycerol release in subcutaneous and omental adipose tissue, respectively (Table 2). Dose response curves were created for both depots for each cow (Figure 3) and the shape of each dose response curve is determined by 2 parameters: \max_{ISO} being the maximal amount of glycerol released above basal glycerol release and $\text{ED}_{50\text{ISO}}$ being the effective dose of ISO needed to elicit the half maximal glycerol release (Table 3). \max_{ISO} was influenced by the size of the adipocytes (volume) and an interaction between both size of the adipocytes and the depot (Table 4, Figure 4). $\text{ED}_{50\text{ISO}}$ was negatively influenced by the size of the adipocytes independent of the adipose depot (Table 4).

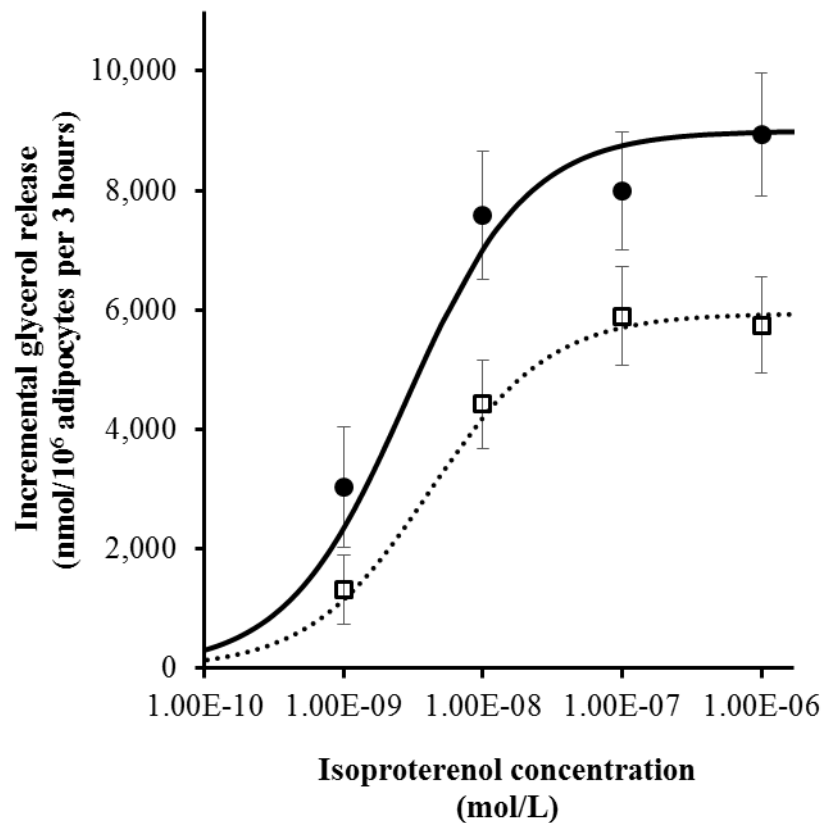


Figure 3: Visualization of the incremental glycerol release of the adipose tissue explants after addition of isoproterenol. Points represent the means of the incremental glycerol release of subcutaneous (●) and omental (□) adipose tissue, and vertical error bars represent the SEM. The lines represent the fitted dose response curves from the nonlinear equation: $\text{Incremental glycerol release} = \frac{\max_{\text{ISO}}}{(1 + 10^{\log \text{ED}_{50\text{ISO}} - \log \text{ISO}})}$ for subcutaneous (—) and omental (...) adipose tissue, with \max_{ISO} being the maximal amount of glycerol released from the explants and $\text{ED}_{50\text{ISO}}$ being the effective dose of isoproterenol needed to elicit the half maximal glycerol release.

Table 3. Model parameters (mean \pm SEM) of the dose response curves of the stimulated and inhibited glycerol release

Item	Subcutaneous	Omental
Isoproterenol stimulated glycerol release ¹		
max _{ISO} (nmol glycerol/10 ⁶ adipocytes per 3 hours)	8,998 \pm 1,307	5,944 \pm 1,124
ED50 _{ISO} (x 10 ⁻⁸ mol/L)	1.08 \pm 0.81	0.71 \pm 0.22
Insulin inhibited glycerol release ²		
min _{INS} (%)	68.8 \pm 4.5	76.8 \pm 5.2
ED50 _{INS} (μ IU/mL)	1.36 \pm 0.51	5.05 \pm 2.35

¹ Calculated as the incremental glycerol release above basal. Dose response curves were created using the PROC NLIN function using the following equation: $\text{Incremental glycerol release} = \frac{\text{max}_{\text{ISO}}}{(1 + 10^{\log \text{ED}_{50\text{ISO}} - \log \text{ISO}})}$. The shape of the dose response curve is determined by 2 parameters: max_{ISO} being the maximal amount of glycerol released from the explants and ED50_{ISO} being the effective dose of isoproterenol needed to elicit the half maximal glycerol release.

² Calculated as percentage of the maximal isoproterenol stimulated glycerol release without insulin. Dose response curves were created using the PROC NLIN function using the following equation: $\% \text{ Inhibited glycerol release} = \text{min}_{\text{INS}} + \frac{(1 - \text{min}_{\text{INS}})}{(1 + 10^{\log \text{INS} - \log \text{ED}_{50\text{INS}}})}$. The shape of the dose response curve is determined by 2 parameters: min_{INS} being the minimal percentage of glycerol released from the explants (representing the maximal inhibitory effect of insulin) and ED50_{INS} being the effective dose of insulin needed to elicit the half maximal inhibitory effect of insulin.

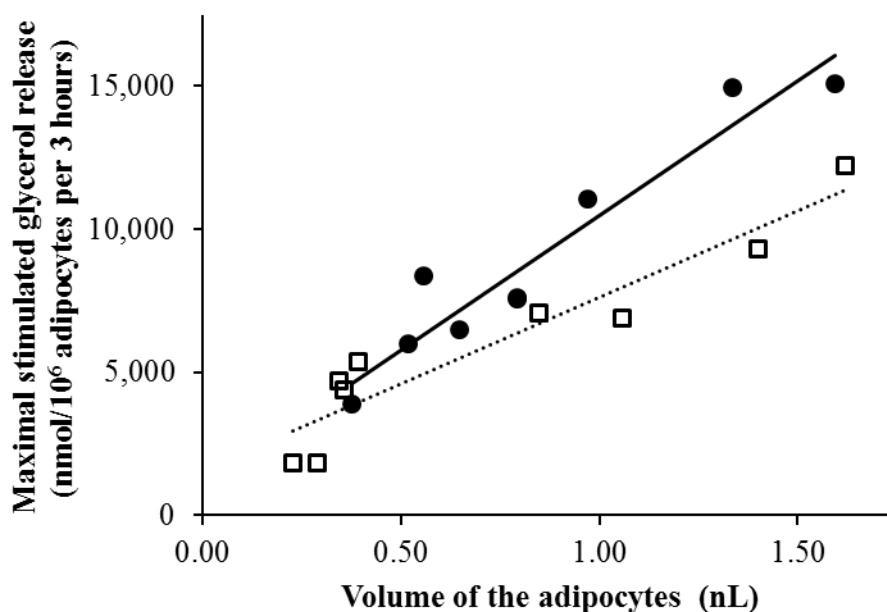


Figure 4: Visualization of the interaction effect of depot and volume of the adipocytes on the maximal isoproterenol stimulated glycerol release (max_{ISO}). Points represent the observations of subcutaneous (●) and omental (□) adipose tissue, and lines represent the linear regression for subcutaneous (—) and omental (...) adipose tissue.

Table 4. Coefficients of the regression models describing the relationship between volume of the adipocytes and adipose depot, and the basal lipolysis and the parameters of the dose response curves of the isoproterenol-stimulated and insulin-inhibited glycerol release

	Basal lipolysis ¹	Isoproterenol-stimulated glycerol release		Insulin-inhibited glycerol release	
		max _{ISO} ²	ED50 _{ISO} ¹	min _{INS} ²	ED50 _{INS} ¹
Intercept	5.39*	1,062	-18.10*	55.09*	-1.26
Volume	1.61*	9,414*	-1.88*	16.28†	1.32
Adipose depot					
Subcutaneous	Referent	Referent	Referent	Referent	Referent
Omental	-0.18	484	0.16	8.91	0.59
Volume * Adipose depot	NS		NS	NS	NS
Volume * subcutaneous		Referent			
Volume * omental		-3,352*			

¹ regression coefficients of the generalized linear mixed model.² regression coefficients of the linear mixed model.* $P < 0.05$; † $0.05 < P < 0.1$; NS non significant.

4.4. Insulin inhibited glycerol release

Addition of insulin inhibited the ISO stimulated glycerol release in both subcutaneous and omental adipose tissue. At maximal insulin concentration, the glycerol release was decreased by 23 to 29% compared to the maximal ISO stimulated glycerol release in omental and subcutaneous adipose tissue, respectively (Table 2). Dose response curves were created for both depots for each cow (Figure 5) and the shape of each dose response curve is determined by 2 factors: min_{INS} being the minimal percentage of glycerol released from the explants and ED50_{INS} being the effective dose of insulin needed to elicit the half minimal glycerol release (Table 3). Min_{INS} was influenced by the volume of the adipocytes (trend) whereas the adipose depot had no effect (Table 4). ED50_{INS} was not influenced by either depot or size of the adipocytes (Table 4).

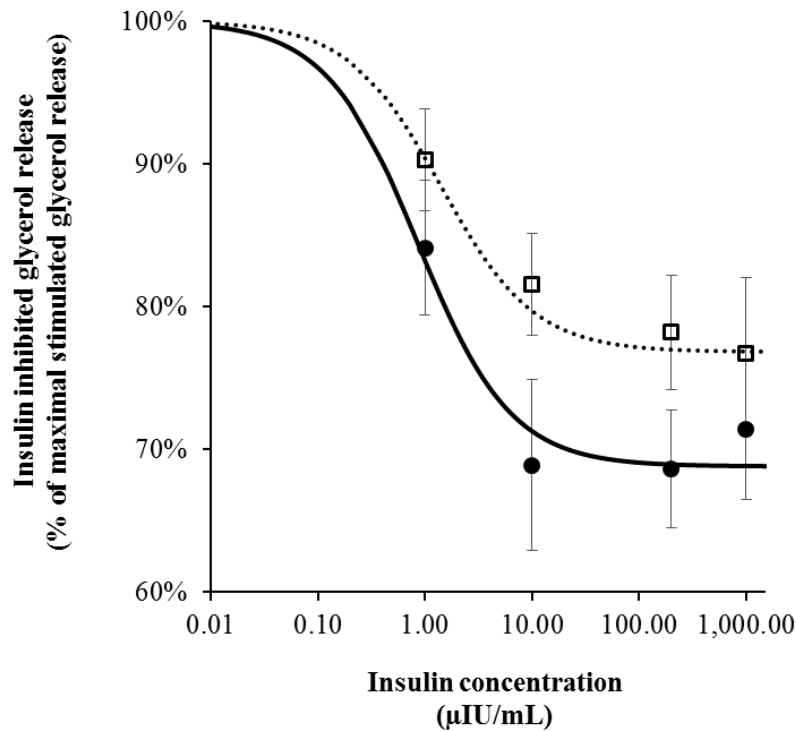


Figure 5: Visualization of the inhibition of the glycerol release of the adipose tissue explants after addition of insulin. Points represent the percentage of the maximal isoproterenol stimulated glycerol release of subcutaneous (●) and omental (□) adipose tissue, and vertical error bars represent the SEM. The lines represent the fitted dose response curves from the nonlinear equation: $\% \text{ Insulin inhibited glycerol release} = \min_{INS} + \frac{(1 - \min_{INS})}{(1 + 10^{\log INS - \log ED50_{INS}})}$ for subcutaneous (—) and omental (...) adipose tissue, with \min_{INS} being the minimal percentage of glycerol released from the explants and $ED50_{INS}$ being the effective dose of insulin needed to elicit the half maximal inhibitory effect of insulin on the lipolytic activity.

5. DISCUSSION

In this study, we investigated the influence of adipocyte size and adipose depot location on the in vitro lipolytic activity of adipocytes in basal conditions and, after addition of different concentrations of catecholamines and insulin in dry dairy cows across a range of BCS. We hypothesized that larger adipocytes from overconditioned cows are more insulin resistant and have higher basal and stimulated lipolytic activity which potentially contributes to excessive fat mobilization in the periparturient period.

5.1. Study design

To avoid the effect of feeding level during the dry period on adipose tissue metabolism, the cows in the present study were fed according to their requirements as reported by De Koster et al. (2015). Explant cultures of adipose tissue were used to reflect as much as possible the in vivo activity of the adipocytes and to preserve paracrine interactions between different cell types in the adipose tissue (Fried and Moustaid-Moussa, 2001). Results of the in vitro

experiments are reported as glycerol release per million of adipocytes rather than per unit of tissue weight to correct for the observed change in cellularity. Overconditioned cows have a lower number of adipocytes per gram adipose tissue, because larger adipocytes from overconditioned cows occupy more volume compared to smaller adipocytes from lean cows. Because the activity of glycerol kinase is negligible in adipocytes, further metabolism of glycerol is impossible and therefore glycerol release is a good indicator for in vitro lipolytic activity. In contrast, released NEFA can be re-utilized in the re-esterification pathway in the adipocytes and therefore NEFA cannot be used as an indicator for in vitro lipolysis (Lafontan, 2012).

5.2. Basal lipolytic activity

Similarly to Gagliostro and Chilliard (1991), in the present study, the basal in vitro lipolytic activity of subcutaneous and omental adipose tissue increased with adipocyte volume meaning that larger fat cells have higher basal lipolytic activity. Pike and Roberts (1984) failed to detect any relationship between adipocyte size and lipolytic activity probably due to the low variability in adipocyte size in their study. Because adipose triglyceride lipase is the main regulator of basal lipolytic activity (Elkins and Spurlock, 2009; Koltes and Spurlock, 2011), we speculate that the activity of this enzyme is upregulated in larger adipocytes. The combination of the greater absolute fat mass and the higher in vitro basal lipolytic activity of large adipocytes suggests that overconditioned cows are continuously exposed to elevated levels of NEFA already before calving. An increased concentration of NEFA in the prepartum period is an important risk factor for the development of fatty liver, ketosis, and other associated diseases (Ospina et al., 2013).

5.3. Isoproterenol stimulated lipolytic activity

Isoproterenol is a non-selective β -agonist and used as a potent stimulator of lipolysis in adipocytes (Lafontan et al., 1997). Maximal effect of lipolytic agents is expected at concentrations between 1 μ M and 10 μ M (Carpene, 2001). In the present study, a logarithmic dilution series of ISO was used to characterize the in vitro dose response curve of adipose tissue. Based on these dose response curves, we conclude that in dairy cows at the end of the dry period, larger fat cells have a higher sensitivity towards lipolytic signals as demonstrated by a

negative influence of size of the adipocytes on $ED50_{ISO}$. This means that, compared to smaller adipocytes, large adipocytes will release more NEFA and glycerol when exposed to a certain amount of ISO *in vitro*. Additionally, the larger fat cells released more glycerol upon maximal stimulation with ISO and the effect of increase in adipocyte volume on max_{ISO} was more pronounced in subcutaneous adipose tissue. As a consequence, we suggest that, during lipolytic conditions, overconditioned cows (which have large adipocytes) will mobilize more fat and will mobilize fat preferentially from the subcutaneous depots. Whereas cows in normal BCS (small adipocytes), will mobilize fat equally from subcutaneous and omental adipose tissue. This might be an explanation for the fact that in some cows surgically corrected for a left displacement of the abomasum (LDA), substantial amounts of fat can be found in the omentum while a relatively low BCS (reflecting subcutaneous adipose tissue) is found (Hostens et al., 2012). Fat cows are more susceptible for LDA (Shaver, 1997) and at the moment of surgical correction for LDA (typically 2 to 3 weeks after calving), the subcutaneous adipose depot of these cows might already be depleted of triglycerides due to extensive mobilization in the immediate postpartum period. In the same study, Hostens et al. (2012) found that the fatty acid profile of circulating NEFA was more comparable to the fatty acid profile of the omental adipose tissue, suggesting that at the moment of surgical correction for LDA, circulating NEFA mainly originate from the abdominal adipose depots. The authors suggested that this might be due to increased blood flow in the abdominal fat depots and differences in metabolic activity between the adipose depots. In addition to both suggestions, we hypothesize that due to the greater mass of the abdominal adipose depot (66.7% of total body fat) compared to the subcutaneous adipose depot (17.9% of total body fat) (De Koster et al., 2015), the former is expected to be a more important contributor to the circulating NEFA.

The immediate postpartum period is characterized by an increase in lipolytic sensitivity and stimuli and a concomitant decrease in antilipolytic sensitivity and stimuli (Bauman and Currie, 1980; McNamara, 1991). During the lipolytic conditions of the immediate postpartum period, large adipocytes will release more NEFA compared to smaller adipocytes. Therefore, overconditioned cows will mobilize enormous amounts of fat, leading to an overflow of NEFA in the liver with associated adverse health effects like ketosis and fatty liver (Drackley, 1999).

5.4. Insulin inhibited lipolytic activity

Insulin inhibits only catecholamine stimulated lipolytic activity and has no effect on basal lipolytic activity (Morimoto et al., 1998). Therefore in the present study, in vitro inhibitory activity of insulin was tested after stimulation of the lipolytic activity of adipocytes with 10^{-6} M ISO. A dilution series of insulin was used to characterize the dose response curve of adipose tissue by non-linear regression to obtain the maximal inhibitory effect of insulin (min_{INS}) and the insulin concentration needed to elicit half maximal effect (ED50_{INS}). Insulin inhibited the ISO stimulated lipolytic activity in a dose dependent manner. We observed a trend for larger adipocytes to be more resistant to the maximal inhibitory effect of insulin. The insulin sensitivity or ED50_{ins} was within the physiological range of insulin and was not influenced by adipocyte size. In vivo results in the same cows demonstrate no effect of overconditioning on insulin response of the fatty acid metabolism while the insulin response of the glucose metabolism was clearly attenuated in overconditioned dry cows (De Koster et al., 2015). Differences between in vivo and in vitro results might be explained by the fact that in vivo results are measured at total body level whereas in vitro results are measured at adipose depot level. In vitro, only lipolytic activity is taken into account whereas in vivo, more metabolic pathways such as β -oxidation, re-esterification, and lipogenesis, play a role in the circulating concentration of NEFA (Roche et al., 2013). Despite the general acceptance that the lipolytic pathway is insulin resistant in overconditioned cows as proposed by Herdt (2000), results of the present study do not support this assumption. We observed a trend for decreased maximal inhibitory effect of insulin on lipolysis in large adipocytes at concentrations well above the normal physiological range. Whereas the half maximal effect of insulin was reached at normal physiological concentrations and was not influenced by fatness of the animals meaning that the antilipolytic effect of insulin in physiological conditions remains intact across a range of BCS. Although min_{ins} and ED50_{ins} differed numerically between the subcutaneous and omental adipose depot, differences were not statistically significant probably due to the low number of cows in the study. The present research indicates some variability in metabolism between adipose depots, therefore further research is warranted to distinguish metabolic properties of the different adipose depots in dairy cows.

6. CONCLUSIONS

Overconditioned dairy cows are destined to excessive mobilization of NEFA and associated adverse health conditions in the periparturient period due to several factors. In the present study, we investigated the role of the adipose tissue in this increased disease susceptibility of overconditioned cows at the end of the dry period. It can be concluded that in overconditioned cows, the absolute amount of fat is greatly increased and therefore, more fat is available to mobilize during lipolytic conditions. Additionally, the adipocytes in the adipose depots of overconditioned cows are enlarged and the in vitro metabolic properties of these large adipocytes are characterized by an increased basal and catecholamine stimulated lipolytic activity. Contrary to the general acceptance, we found no clear evidence for a state of insulin resistance of the antilipolytic effect of insulin in large adipocytes in vitro. Results from the present in vitro studies suggest that overconditioned cows will mobilize fat preferentially from the subcutaneous depots. Whereas cows in normal BCS, will mobilize fat equally from the subcutaneous and omental adipose tissue. However more research is needed to identify the metabolic properties of the different adipose depots of dairy cows.

7. ACKNOWLEDGMENTS

This research was funded by the Special Research Fund of Ghent University, grant number 01D28410. The authors wish to thank Isabel Lemahieu and Petra Van Damme for excellent technical assistance in the lab.

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CHAPTER 7

Adipokines in dairy cows

CHAPTER 7.1

Influence of adipocyte size and adipose depot on the expression of adipokines in dairy cows at the end of pregnancy

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2016. Influence of adipocyte size and adipose depot on the expression of adipokines in dairy
cows at the end of pregnancy. (In preparation)*

1. ABSTRACT

The aim of this study was to determine the mRNA expression of adipokines (adiponectin, leptin, IL6 and TNF) and CD14 in different adipose depots from cows with a variable body condition score (from normal conditioned to overconditioned) at the end of the dry period. We hypothesized that the expression of these adipokines and of the macrophage-specific marker CD14, would depend on the size of the adipocytes and on the localization of the adipose depot. Expression of the different genes was determined by quantitative reverse transcriptase real-time PCR (RT-qPCR) in subcutaneous, omental, mesenteric, perirenal and intrapelvic adipose tissue samples taken immediately after euthanasia of ten dairy cows at the end of pregnancy. Expression of *LEPT* (leptin), *CD14*, *IL6* and *TNF* was positively associated with adipocyte size, while no association could be detected between *ADIPOQ* (adiponectin) and the size of the adipocytes. The increased expression of *CD14* might indicate infiltration of macrophages in the adipose tissue of overconditioned cows. The expression of the different adipokines was clearly different between the different adipose depots. In general, the expression of pro-inflammatory adipokines (*LEPT* and *IL6*) and *CD14* was greater in the internal compared with the subcutaneous adipose depots. In conclusion, the results of the present study indicate a pro-inflammatory state of adipose depots with large adipocytes in cows at the end of pregnancy which might induce a dysregulation of inflammatory processes and contribute to metabolic and infectious disorders in the periparturient period.

2. INTRODUCTION

Important physiological changes occur during the transition period of dairy cows, being the last three weeks before calving and the first three weeks of lactation (Drackley, 1999). Despite much progress made in the knowledge of the intermediary metabolism of dairy cows during the transition period, 30 to 50 % of the dairy cows suffer from one or more disorders in the transition period (Leblanc, 2010). Different risk factors have been identified in the development of transition cow disorders among which excessive mobilization of body fat is generally established (Drackley, 1999; McArt et al., 2013; Roche et al., 2013). Recent research indicates that a dysregulation of inflammatory processes in the periparturient dairy cow may contribute

to the development of infectious and metabolic disorders (Bertoni et al., 2008; Trevisi et al., 2012; Bradford et al., 2015).

There are different underlying reasons for the dysregulation of inflammatory processes in the periparturient dairy cow, which have been reviewed by Sordillo and Raphael (2013) and Bradford et al. (2015). The adipose tissue is considered to have a causative role in the dysregulation of inflammatory processes in the periparturient period (Vernon, 2002; Drackley et al., 2005; Loores et al., 2007). Obesity in humans is associated with a chronic pro-inflammatory state, known as a metabolically triggered inflammation or metaflammation (Hotamisligil, 2006). The obese adipose tissue, infiltrated with macrophages, is the origin of this inflammation and makes obese people more vulnerable for the development of insulin resistance, type 2 diabetes mellitus and cardiovascular disease (Despres and Lemieux, 2006; Cornier et al., 2008). Especially the excessive accumulation of abdominal or visceral fat is considered to be detrimental for humans, due to metabolic and functional differences between the subcutaneous and abdominal adipose depot (Despres and Lemieux, 2006). Large visceral adipocytes demonstrate a greater lipolytic activity, lower insulin sensitivity and a dysregulated production of adipokines characterized by greater pro- and lower anti-inflammatory levels of adipokines (Despres and Lemieux, 2006). However, metabolic properties of the different adipose depots are very species-specific. In horses, the adipose depot around the nuchal ligament is associated with a pro-inflammatory expression profile and negative health consequences (insulin resistance, laminitis) (Burns et al., 2010; Bruynsteen et al., 2013). Whereas in obese cats, pro-inflammatory genes were especially expressed in the subcutaneous adipose depot (Van de Velde et al., 2013).

Overconditioned dairy cows are known to be at an increased risk for the development of different transition problems. This is, in the first place, due to excessive release of non-esterified fatty acids (NEFA) from the adipose tissue in the immediate postpartum period (Roche et al., 2009). However, it is not known whether the adipose tissue of overconditioned cows is also responsible for a dysregulated production of adipokines, whether there is a depot-specific difference in the production of adipokines and whether there is an infiltration of macrophages in the adipose tissue of overconditioned dairy cows.

The aim of the present study was to determine the mRNA expression of adipokines (adiponectin, leptin, IL6 and TNF) and CD14 in different adipose depots from cows with a variable BCS (from normal conditioned to overconditioned) at the end of the dry period. We hypothesized that the expression of the different adipokines and of the macrophage-specific marker CD14 depends on the size of the adipocytes and differs between different adipose depots.

3. MATERIALS AND METHODS

All experimental procedures were approved by the ethical committee of the Faculty of Veterinary Medicine (EC2010-/149, Ghent University, Belgium).

3.1. Study design

Ten clinically healthy, pregnant Holstein Friesian dairy cows (upcoming parity 2 to 5) were selected at the beginning of the dry period based on BCS according to the scale of Edmonson et al. (1989). Five animals were considered to have a normal BCS (BCS 2.5 - 3.5) and 5 animals were considered to be overconditioned (BCS 3.75 - 5). During the dry period (starting approximately 7 weeks before the expected parturition date), animals were weekly monitored by assessment of their BCS. A more detailed description of the study design can be found in De Koster et al. (2015).

Cows were euthanized 10 to 13 days before the expected parturition date at the Department of Morphology (Faculty of Veterinary Medicine, Ghent University, Belgium). Cows were stunned with a captive bolt gun and exsanguinated by transecting the carotid arteries and the jugular veins. Immediately after euthanasia, adipose tissue samples were collected from the subcutaneous, omental, mesenteric, perirenal and intrapelvic adipose depots. Subcutaneous adipose tissue samples were taken from the adipose tissue located in the fossa ischiorectalis. Omental adipose tissue samples were taken from the omentum majus at the right side at the level of the pylorus, halfway between the cranial and caudal rim of the omentum. Mesenteric adipose tissue samples were taken 15 cm proximal from the jejunum. Perirenal adipose tissue samples were taken from the caudal end of the right kidney. Samples for mRNA expression were immediately snap frozen in liquid nitrogen and stored at -80°C until processing. Samples for histology were fixed in 4 % buffered formaldehyde (pH 7.4) at room temperature for 24 h,

subsequently dehydrated in a tissue processor (Microm STP 420D, Prosan, Merelbeke, Belgium) and embedded in paraffin blocks using an embedding station (Microm EC 350-1 and Microm EC 350-2, Prosan, Merelbeke, Belgium). After euthanasia of the cows, calves were excised by a left flank incision and euthanized by intravenous injection of sodium pentobarbital (Release®, 90 mg/kg).

3.2. Histology

Two sections (8 µm thick) of each adipose tissue sample were made using a microtome (Microm HM 360 microtome, Prosan, Merelbeke, Belgium) and mounted on slides. After staining with haematoxylin (Haematoxylin, C.I.75290, Merck KGaA, Darmstadt, Germany) and eosin (Eosin yellow, C.I.45380, VWR international bvba, Leuven, Belgium), five images were taken at random on each of the sections at 100 x magnification using a microscope (Olympus BX 61, Olympus, Belgium). On each image, the area of 10 adipocytes was determined using Cell F software (Olympus, Belgium). To avoid that the same adipocyte was measured twice, the two sections were made at least 200 µm apart.

3.3. Primer design, reference gene selection and RT-qPCR

Primers for adiponectin, C1Q and collagen domain containing (*ADIPOQ*, NCBI gene ID: 282865), leptin (*LEP*, NCBI gene ID: 280836), CD14 molecule (*CD14*, NCBI gene ID: 281048) and tumor necrosis factor (*TNF*, NCBI gene ID: 280943) were designed using Primer3Plus (Untergasser et al., 2007), based on DNA sequences in Genbank. Secondary structures were avoided with Mfold (Zuker, 2003). Primers for interleukin 6 (*IL6*, NCBI gene ID: 280826) were used as described by Vailati Riboni et al. (2015). Based upon previous research, six candidate reference genes were selected: MARVEL domain containing 1 (*MARVELD1*, NCBI gene ID: 616867), eukaryotic translation initiation factor 3 subunit K (*EIF3K*, NCBI gene ID: 515326), LDL receptor related protein 10 (*LRP10*, NCBI gene ID: 515494), actin beta (*ACTB*, NCBI gene ID: 280979), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*, NCBI gene ID: 2870222) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*, NCBI gene ID: 281229) (Goossens et al., 2005; Saremi et al., 2012). All primers were ordered from Integrated DNA Technologies (IDT, Leuven, Belgium). Primer and amplicon information can be found in Table 1.

Table 1. Characteristics of the primers used for RT-qPCR.

Gene	Sequence 5'-3'	Product size (bp)	Ta (°C)	R ²	Efficiency (%)	Genbank accession number or reference
<i>MARVELD1</i>		100	63.0	0.998	102.35	Saremi et al., 2012
Forward:	GGCCAGCTGTAAGATCATCACA					
Reverse:	TCTGATCACAGACAGAGCACCAT					
<i>EIF3K</i>		125	59.5	0.999	102.60	Saremi et al., 2012
Forward:	CCAGGCCCCACCAAGAAGAA					
Reverse:	TTATACCTTCCAGGAGGTCCATGT					
<i>LRP10</i>		139	61.0	0.999	102.30	Saremi et al., 2012
Forward:	CCAGAGGATGAGGACGATGT					
Reverse:	ATAGGGTTGCTGTCCCTGTG					
<i>ACTB</i>		87	58.0	0.999	96.25	Goossens et al., 2005
Forward:	CCTCACGGAACGTGGTTACA					
Reverse:	TCCTTGATGTCACGCACAATTT					
<i>HPRT1</i>		154	58.0	0.999	102.25	Goossens et al., 2005
Forward:	TGCTGAGGATTTGGAGAAGG					
Reverse:	CAACAGGTCGGCAAAGAAGT					
<i>YWHAZ</i>		120	60.0	0.999	101.90	Goossens et al., 2005
Forward:	GCATCCCACAGACTATTTCC					
Reverse:	GCAAAGACAATGACAGACCA					
<i>CD14</i>		154	58.0	0.998	103.05	NM_174008.1
Forward:	AGCCCTCCAGCACCAAAA					
Reverse:	TTCATTCCTCTTCCCTCTCTTCC					
<i>ADIPOQ</i>		100	64.0	0.999	101.1	NM_174742.2
Forward:	CCACCTTCACAGGCTTCCTTCT					
Reverse:	GTTCTTTCACTTTGTGCTGCTTGG					
<i>LEP</i>		148	64.0	0.999	101.9	NM_173928.2
Forward:	CTGTGGCTTTGGCCCTATCTGT					
Reverse:	CAGTGACCCTCTGTTTGGAGGAG					
<i>IL6</i>		100	62.0	0.997	102.4	Vailati Riboni et al., 2015
Forward:	CCAGAGAAAAACCGAAGCTCTCAT					
Reverse:	CCTTGCTGCTTTCACACTCATC					
<i>TNF</i>		95	66.0	0.997	98.6	NM_173966.3
Forward:	TGCCTGCTGACGGGCTTTA					
Reverse:	GCTGATGGTGTGGGTGAGGA					

Total RNA was isolated using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The samples were homogenized in Purezol using a Tissue Ruptor (Qiagen, Antwerp, Belgium). After RNA isolation, a DNase treatment with 6 μ L RQ1 RNase-Free DNase (1 U/ μ L, Promega, Leiden, Netherlands) and 3 μ L RQ1 DNase 10x Reaction Buffer (Promega, Leiden, Netherlands) was performed for 15 min at room temperature. Afterwards, RNA was purified by spin-column centrifugation (Amicon Ultra-0.5 centrifugal filters, Merck Millipore, Billerica, MA, USA). Genomic DNA contamination of the samples was verified by a minus reverse transcription control PCR with *LRP10* primers. Quantity and purity (OD260/280 ratio 1.91 - 2.16) of RNA were evaluated with the Nanodrop ND-1000 spectrophotometer (Nanodrop

Products, Thermo Scientific, Wilmington, DE, USA). Additionally, RNA quality was verified by visualization of the ribosomal bands on a 2% agarose gel. When RNA quality was sufficient, RNA (max 1 µg) was converted to cDNA using the Improm-II cDNA synthesis kit (Promega, Madison, WI, USA). First, RNA was mixed with 0.5 µL oligo(dT) (10 µM, Integrated DNA Technologies, Leuven, Belgium) and 0.5 µL random hexamers (10 µM, Integrated DNA Technologies, Leuven, Belgium). The mix was heated during 5 min at 70°C followed by cooling on ice for 5 min. Afterwards, 4 µL Improm-II 5x reaction buffer, 2.4 µL MgCl₂ (25 mM), 1 µL dNTP Mix (10 mM each; Bioline Reagents, London, UK) and 1 µL Improm-II reverse transcriptase (20 U/µL) were added. This mix was incubated for 5 min at 25°C (annealing), 60 min at 42°C (synthesis of cDNA) and 15 min at 72°C (RT inactivation). cDNA (10 fold diluted) was verified by a control PCR with *LRP10* primers.

All PCR reactions were performed in a reaction volume of 10 µL on the Eppendorf Mastercycler PCR system (Eppendorf, Rotselaar, Belgium) with 0.5 U TEMPase Hot Start DNA Polymerase (Ampliqon, Odense, Denmark), 1 µL reaction buffer (10x, Roche, Brussels, Belgium), 0.2 µL dNTP (10 mM each, Bioline Reagents, London, UK), 1 µL primer mix (5 µM forward and reverse primer, Integrated DNA Technologies, Leuven, Belgium) and 2 µL sample. The PCR program consisted of an initial denaturation step (15 min at 95°C) followed by 40 cycles of 15 sec at 95°C, 30 sec at the annealing temperature of the primer and 30 sec at 72°C with a final elongation of 2 min at 72°C. The PCR products were visualized on a 2% agarose gel.

The quantitative PCR reactions were performed using KAPA SYBR FAST qPCR kit master mix (Kapa Biosystems, Wilmington, MA, USA) in a reaction volume of 10 µL containing 5 µL KAPA SYBR FAST, 1 µL primers (5 µM forward and reverse) and 2 µL cDNA sample (10 fold diluted). The PCR was conducted on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and started at 95°C for 4 min, followed by 40 cycles of 20 sec at 95°C and 40 sec at the annealing temperature of the primer with detection of fluorescence. A melting curve was generated by heating the samples from 70°C tot 95°C in steps of 0.5°C for 5 sec with fluorescence detection. In each run, a serial dilution (6-points, 4-fold dilution) of a cDNA sample and a no template control were included to determine the

efficiency and R^2 . All reactions were performed in duplicate. The stability of the reference genes was checked using GeNorm (Vandesompele et al., 2002). The C_q values of the target genes were converted to raw data based on the efficiency of the qPCR after which they were normalized with the geometric mean of the three most stable reference genes (*MARVELD1*, *EIF3K*, *LRP10*) as described by Erkens et al. (2006). Non-detects (*CD14* n = 3 samples; *IL6* n = 4 samples; *TNF* n = 7 samples) were imputed using the fully conditional specification method using the PROC MI function (McCall et al., 2014). Interpretation of the results were similar with or without the imputed values for the non-detects.

3.4. Statistical analyses

Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Descriptive statistics (PROC MEANS) are expressed as mean \pm SEM unless otherwise indicated. Normality of the variables (PROC UNIVARIATE) was checked using the Kolmogorov-Smirnov test. For the comparison of the size of the adipocytes between depots, diameter and area of the adipocytes were fitted as dependent variable in a model, depot was included as independent variable and cow was included as random factor. Because diameter and area were lognormally distributed, the model was fitted using a generalized linear mixed model (PROC GLIMMIX) using the DIST = LOGNORMAL statement. Pairwise comparisons between depots were checked using the LSMEANS statement (Tukey post-hoc test). To determine the significant effects on mRNA expression of the different genes, a model was built with the expression of the individual genes as dependent variable, depot, average adipocyte area per depot and the interaction between depot and adipocyte area were included as independent variables and cow was included as random factor. Interaction terms were left out the model if not significant ($P > 0.10$). Because the expression of the genes was lognormally distributed, the model was fitted using a generalized linear mixed model (PROC GLIMMIX) using the DIST = LOGNORMAL statement. Pairwise comparisons between depots were checked using the LSMEANS statement (Tukey post-hoc test).

To determine the relationship between the expression of the different adipokines, a mixed model (PROC MIXED) was built with the mRNA expression of the each gene (after log transformation to yield normal distribution) as dependent variable and the mRNA expression

of the other genes (after log transformation to yield normal distribution) as independent variable. Depot was included as random factor within the individual cow, however the random factor was not significant (COVTEST ZEROG) and left out to model to avoid over specification of the model. Regression coefficients of the different models are presented to demonstrate the relationship (positive or negative) between the expression of the individual adipokines. Pearson correlation coefficients were calculated between the average area of the adipocytes per depot and the BCS (average BCS during the dry period). Significance and tendency were declared at $P < 0.05$ and $0.05 < P < 0.1$, respectively.

4. RESULTS

Mesenteric adipocytes were significantly smaller, whereas intrapelvic adipocytes were significantly larger compared with the other 3 depots (subcutaneous, omental and perirenal) (Table 2). Pearson correlation coefficients between the average BCS of the cows during the dry period and the adipocyte size were strong and significantly positive in all the depots: subcutaneous adipose tissue ($r = 0.97$; $P < 0.05$), mesenteric adipose tissue ($r = 0.80$; $P < 0.05$), omental adipose tissue ($r = 0.85$; $P < 0.05$), intrapelvic adipose tissue ($r = 0.92$; $P < 0.05$) and perirenal adipose tissue ($r = 0.90$; $P < 0.05$).

Table 2. Adipocyte size (Mean \pm SEM) by adipose depot.

Parameter	Adipose tissue depot				
	Subcutaneous	Mesenteric	Omental	Intrapelvic	Perirenal
Area (μm^2)	10,475 \pm 123 ^a	8,500 \pm 91 ^b	10,383 \pm 135 ^a	11,466 \pm 125 ^c	11,087 \pm 173 ^a
Diameter (μm)	118.65 \pm 0.66 ^a	107.93 \pm 0.48 ^b	117.31 \pm 0.66 ^a	123.99 \pm 0.61 ^c	120.65 \pm 0.83 ^a

^{abc} Means within a row with different superscript letters differ significantly ($P < 0.05$).

Expression of the different genes in the different adipose tissue depots is represented in Table 3 and Figure 1 and relationship between the size of the adipocytes and the expression is represented in Table 4 and Figure 2. Expression of *ADIPOQ* was not influenced by adipocyte size ($P > 0.05$) but significantly influenced by adipose depot ($P < 0.05$), with lower expression in the subcutaneous depot when compared with the omental and intrapelvic depots. Expression of *LEPT* was significantly different between the adipose depots ($P < 0.05$) with lower expression in the subcutaneous and omental depots compared with the intrapelvic depot. There was a trend ($0.05 < P < 0.1$) for lower *LEPT* expression in the subcutaneous depot compared

with the mesenteric and perirenal depots. The effect of adipocyte size on the expression of *LEPT* was dependent on the adipose depot (interaction effect). The expression of leptin was greater in larger adipocytes and this was more pronounced in the mesenteric adipose depot. Expression of *CD14*, *IL6* and *TNF* was greater in larger adipocytes ($P < 0.05$). Furthermore, expression of *CD14* was different between the adipose depots ($P < 0.05$) with greater expression in the omental adipose depot when compared with the intrapelvic, mesenteric and subcutaneous depots. There was furthermore a trend for greater expression in the omental adipose depot when compared with the perirenal adipose depot. Expression of *IL6* was influenced by the adipose depots ($P < 0.05$) with lower expression in the subcutaneous depot when compared with the other depots. Expression of *TNF* was lower in the intrapelvic depot when compared with the other depots.

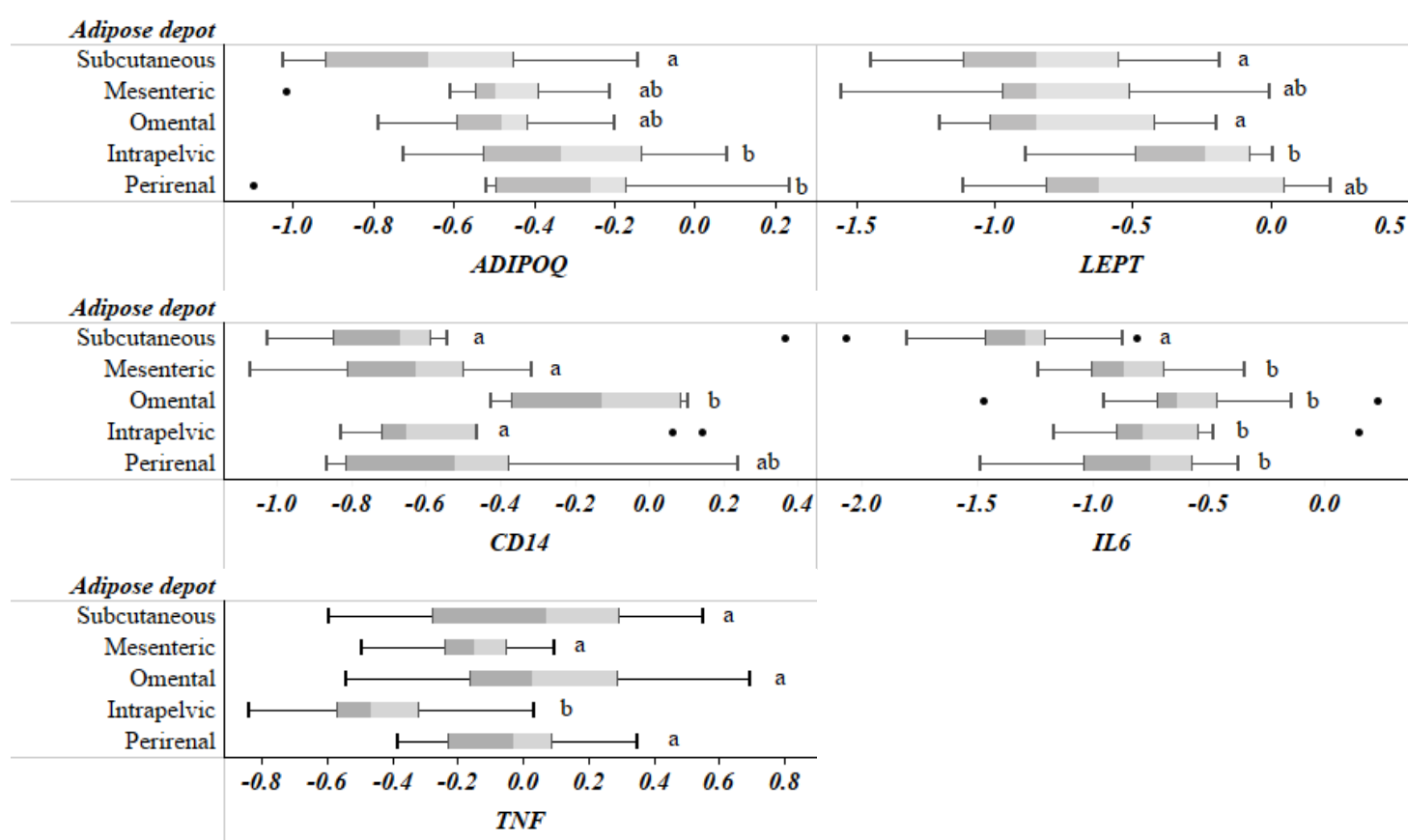


Figure 1: mRNA expression¹ of *ADIPOQ*, *LEPT*, *CD14*, *IL6* and *TNF* in different adipose tissue depots².

^{ab} Depots with different letters within a gene differ significantly ($P < 0.05$).

¹ Data are expressed as relative mRNA abundance after normalization with the reference genes (*MARVELD1*, *EIF3K* and *LRP10*) and log transformation.

The relationship between the expression of the different genes is represented by the coefficients of the linear mixed model (Table 4) and the scatterplot (Figure 3). Within the different adipose depots, there was a positive relationship between *ADIPOQ* and *LEPT*, a negative relationship between *ADIPOQ* and *CD14*, a negative relationship between *ADIPOQ* and *TNF*, a positive relationship between *CD14* and *IL6* and a positive relationship between *CD14* and *TNF*.

Table 3. mRNA expression (Mean \pm SEM) of *ADIPOQ*, *LEPT*, *CD14*, *IL6* and *TNF* in different adipose tissue depots.

Gene ¹	Adipose tissue depot				
	Subcutaneous	Mesenteric	Omental	Intrapelvic	Perirenal
<i>ADIPOQ</i>	0.275 \pm 0.063 ^a	0.344 \pm 0.044 ^{ab}	0.351 \pm 0.047 ^{ab}	0.561 \pm 0.111 ^b	0.620 \pm 0.141 ^b
<i>LEPT</i>	0.225 \pm 0.066 ^a	0.262 \pm 0.091 ^{ab}	0.247 \pm 0.069 ^a	0.589 \pm 0.096 ^b	0.581 \pm 0.186 ^{ab}
<i>CD14</i>	0.410 \pm 0.214 ^a	0.254 \pm 0.037 ^a	0.786 \pm 0.115 ^b	0.433 \pm 0.143 ^a	0.492 \pm 0.169 ^{ab}
<i>IL6</i>	0.061 \pm 0.015 ^a	0.179 \pm 0.038 ^b	0.403 \pm 0.155 ^b	0.302 \pm 0.125 ^b	0.190 \pm 0.037 ^b
<i>TNF</i>	1.373 \pm 0.318 ^a	0.733 \pm 0.085 ^a	1.568 \pm 0.443 ^a	0.449 \pm 0.094 ^b	1.00 \pm 0.172 ^a

^{ab} Means within a row with different superscript letters differ significantly ($P < 0.05$).

¹ Data are expressed as relative mRNA abundance after normalization with the reference genes (*MARVELD1*, *EIF3K*, *LRP10*).

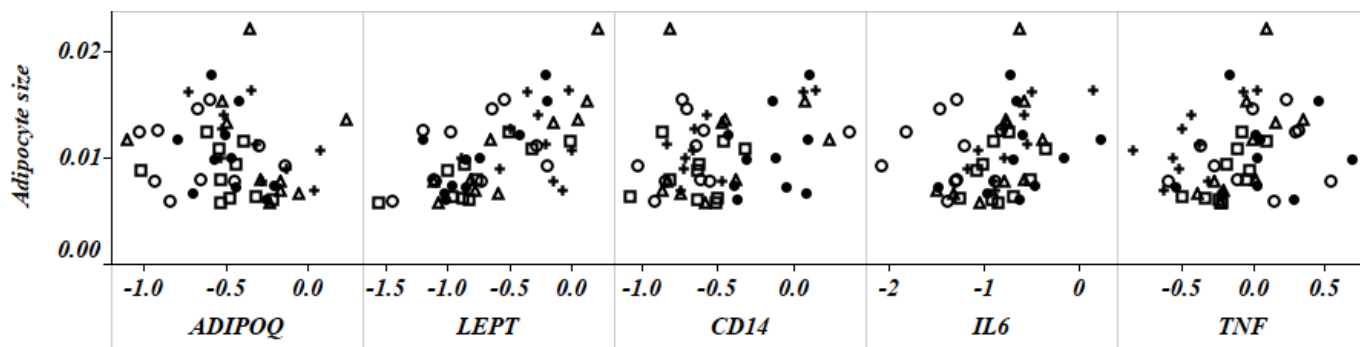


Figure 2: Scatterplot between the mRNA expression¹ of the different adipokines (*ADIPOQ*, *LEPT*, *IL6* and *TNF*) and *CD14* and adipocyte size throughout the different adipose depots (5 adipose depots² in 10 cows).

¹ Data are expressed as relative mRNA abundance after normalization with the reference genes (*MARVELD1*, *EIF3K* and *LRP10*) and log transformation.

² Subcutaneous adipose tissue = ○; mesenteric adipose tissue = □; omental adipose tissue = ●; intrapelvic adipose tissue = +; perirenal adipose tissue = Δ.

Table 4. Coefficients of the regression models describing the relationship between the relative expression of the different genes and the area of the adipocytes.

Item	<i>ADIPOQ</i>	<i>LEPT</i>	<i>CD14</i>	<i>IL6</i>	<i>TNF</i>
Area (mm ²) ¹	-22.79	102.87 *	63.64 *	78.44 †	66.50 *
<i>ADIPOQ</i> ²		0.41 *	- 0.26 *	0.02	-0.27 *
<i>LEPT</i> ²	0.89 *		- 0.08	0.11	-0.31 †
<i>CD14</i> ²	-0.47 *	0.05		0.31 *	0.37 *
<i>IL6</i> ²	-0.01	0.14	0.47 *		0.22
<i>TNF</i> ²	-0.46 *	-0.15	0.31 *	0.14	

¹ regression coefficients of the generalized linear mixed model.² regression coefficients of the mixed model.* $P < 0.05$; † $0.05 < P < 0.1$.

5. DISCUSSION

Overconditioned cows are prone to the development of different metabolic and infectious disorders, which is known as the fat cow syndrome (Morrow, 1976; Roche et al., 2009). The cows in the present study were fed according to their requirements and did not gain nor lose BCS during the dry period (De Koster et al., 2015). The excessive BCS in the overconditioned cows was most probably due to a prolonged positive energy balance at the end of the previous lactation. Different papers demonstrated a dysregulated inflammatory reaction in overconditioned cows. Cows with high BCS had greater concentrations of markers for oxidative stress, decreased functional properties of circulating mononuclear cells (lower IFN-gamma and IgM secretion) and greater plasma levels of tumor necrosis factor (TNF) (Bernabucci et al., 2005; Lacetera et al., 2005; O'Boyle et al., 2006). The aim of the present study was to describe the potential role of the adipose tissue in creating a pro-inflammatory state in overconditioned cows. More specifically, we aimed to determine the association between the size of the adipocytes, the location of the adipose depot and the mRNA expression for adiponectin, leptin, CD14, IL6 and TNF in adipose tissue samples taken from dairy cows with a variable BCS at the end of pregnancy.

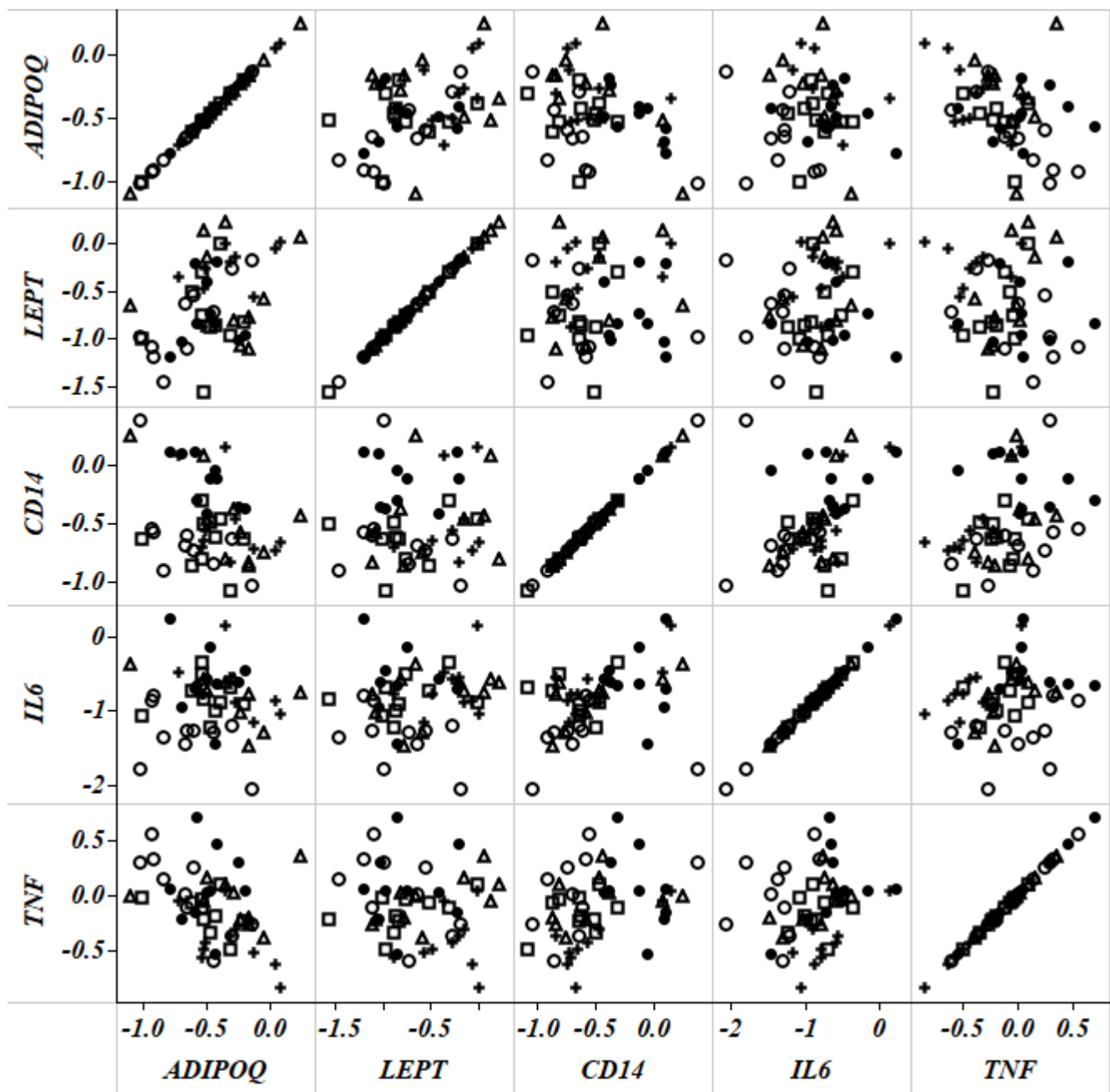


Figure 3: Scatterplot matrix of the mRNA expression¹ of the different adipokines (*ADIPOQ*, *LEPT*, *IL6* and *TNF*) and *CD14* and adipocyte size throughout the different adipose depots (5 adipose depots² in 10 cows).

¹ Data are expressed as relative mRNA abundance after normalization with the reference genes (*MARVELD1*, *EIF3K* and *LRP10*) and log transformation.

² Subcutaneous adipose tissue = ○; mesenteric adipose tissue = □; omental adipose tissue = ●; intrapelvic adipose tissue = +; perirenal adipose tissue = △.

5.1. Adipocyte size

Overconditioned cows have larger adipocytes as demonstrated by the strong correlation between BCS and adipocyte size in every adipose depot. The latter supports the observation that accumulation of lipids in adult cows is mainly mediated via hypertrophy of the adipocytes instead of via hyperplasia (Hood and Allen, 1973; Eguinoa et al., 2003). Similar to Akter et al. (2011), the largest adipocytes in the present study were found in the retroperitoneal adipose depot (intrapelvic and perirenal adipose depots). Differences in adipocyte size between depots are related to depot-specific differences in adipose tissue metabolism: accumulation and/or release of lipids. In the present study, the different adipocyte size between depots is probably a reflection of a different lipogenic activity in different adipose depots at the end of the previous lactation due to the fact that cows were not yet metabolically triggered to release large amounts of NEFA at the moment of euthanasia. Kahn et al. (2013) suggested that lipogenesis is mainly genetically controlled (by transcription of key lipogenic proteins), while lipolysis is regulated by posttranslational modifications. As a consequence, expression of lipogenic genes reflects the lipogenic potential of an adipose depot (Ji et al., 2014b). Ji et al. (2014a) demonstrated different expression of lipogenic genes between the subcutaneous, omental and mesenteric adipose depots in nonpregnant-nonlactating cows. The results of the latter study are consistent with a greater lipogenic potential in subcutaneous compared with mesenteric adipose tissue (Ji et al., 2014a). Which might explain the smaller adipocyte size in the mesenteric adipose tissue observed in the present study.

5.2. Influence of adipocyte size and adipose depot on *ADIPOQ* and *LEPT* expression

Adipocytes are the main source of circulating adiponectin and leptin (Chilliard et al., 2005; Singh et al., 2014). Both adipokines play an important role in the regulation of energy homeostasis. Expression of *LEPT* in the adipose tissue is strongly related to the serum levels of leptin and is determined by chronic and acute regulatory mechanisms (Drackley et al., 2006). Long term effects are regulated by body fatness while short term effects are regulated by feed intake and energy balance. During negative energy balance and feed deprivation, leptin concentrations decrease (Chilliard et al., 2005). Cows in the present study were in positive energy balance and not deprived of feed thus leptin levels were mainly determined by body

fatness. This might explain the positive association between leptin and the adipocyte size as described previously (Chilliard et al., 2005).

Expression of *LEPT* was furthermore greater in the intrapelvic adipose depot. Expression of *ADIPOQ* was greater in the retroperitoneal adipose depots (intrapelvic and perirenal adipose tissue) when compared with the subcutaneous adipose depot. Similarly, in the study of Saremi et al. (2014), *LEPT* and *ADIPOQ* expression were numerically greater in the retroperitoneal adipose depot. It should be noted that the expression of *ADIPOQ* is not correlated with serum concentrations of adiponectin. Adiponectin production is mainly controlled by post-transcriptional factors (Lemor et al., 2009; Singh et al., 2014). Therefore, the lack of association between adipocyte size and *ADIPOQ* expression in the present study does not exclude a possible association between adiponectin concentration and fatness of the animals. Indeed, a negative correlation between serum adiponectin concentration and BCS has been demonstrated (Singh et al., 2014). Besides its well described role in the regulation of feed intake, leptin exerts pro-inflammatory effects by activating the inflammatory cascade in immune cells (Ingvarsen and Boisclair, 2001; Paz-Filho et al., 2012). Contrary, adiponectin exerts anti-inflammatory effects (Yamauchi and Kadowaki, 2008) and these anti-inflammatory properties have been confirmed in cattle after an in vitro LPS challenge of monocytes (Kabara et al., 2014). The unchanged expression of *ADIPOQ* and the increased expression of *LEPT* in adipose depots with large adipocytes might suggest a pro-inflammatory state of these adipose depots. Leptin and adiponectin exert both insulin sensitizing effects mainly by activating adenosine monophosphate-activated protein kinase (AMPK) which increases fatty acid oxidation in skeletal muscle. This will lead to decreased triglyceride accumulation which is associated with improved insulin sensitivity (Kahn and Flier, 2000; Kadowaki and Yamauchi, 2005). However, the insulin sensitizing effect of leptin and adiponectin requires further investigation in dairy cows.

5.3. Influence of adipocyte size and adipose depot on *CD14* and *IL6* expression

Besides adipocytes, the white adipose tissue contains pre-adipocytes, fibroblasts, endothelial cells, T lymphocytes and macrophages (Heilbronn and Campbell, 2008; Surmi and Hasty, 2008). Obesity in humans is associated with an increased infiltration of adipose tissue

macrophages (ATM) and a subsequent increased production of pro-inflammatory cytokines (Heilbronn and Campbell, 2008). Expression of *CD14* in the adipose tissue has been used as a marker that correlates with the number of ATM (Bruun et al., 2005; Fjeldborg et al., 2014). In cows, *CD14* is used as a marker to identify macrophages and monocytes by immunohistochemistry and flow cytometry (Oliveira and Hansen, 2008; Contreras et al., 2015). Despite the fact that *CD14*, although to a lesser extent (20 to 50 times lower), is also expressed in bovine neutrophils (Ibeagha-Awemu et al., 2008), *CD14* expression in the adipose tissue was assumed to be associated with the number of ATM.

In dairy cows, Akter et al. (2012) did not detect a meaningful infiltration of macrophages in adipose tissue sampled from dairy cows with a moderate BCS during the first months of lactation. Presence of ATM was greater in biopsies taken from overconditioned heifers and steers (Akter et al., 2012). Although the number of ATM was limited, a positive correlation between the number of macrophages and the size of the adipocytes was demonstrated (Akter et al., 2012). In the present study, the expression of *CD14* was detected in all depots suggesting presence of ATM in all depots. Furthermore, the expression of *CD14* was positively associated with the size of the adipocytes which suggests an increased number of ATM in adipose depots with larger adipocytes. Expression of *CD14* was significantly greater in omental adipose tissue. Similarly, Akter et al. (2012) detected lower amounts of ATM in subcutaneous versus visceral adipose tissue and Contreras et al. (2015) found a marked infiltration of ATM in subcutaneous and omental adipose tissue of dairy cows with a left displacement of the abomasum (DA). Especially the omental adipose tissue of the DA cows was infiltrated with ATM which were localized in aggregates similar to the crown like structures observed in human obesity (Cinti et al., 2005; Contreras et al., 2015). The role of these ATM in the development of a pro-inflammatory state, depends on the polarization of the ATM in the adipose tissue (Contreras et al., 2015). Classically activated ATM (M1) are induced by inflammatory stimuli (TNF, LPS) and upon activation, the M1 macrophages produce large amounts of pro-inflammatory cytokines. Alternatively activated ATM (M2) are induced by exposure to IL-4 and IL-13 and produce anti-inflammatory cytokines (IL-10). Classical activated ATM are responsible for inflammation and have high microbicidal activity while alternatively activated ATM are

responsible for the resolution of inflammation and tissue repair (Lumeng et al., 2007; Surmi and Hasty, 2008; Olefsky and Glass, 2010). In the present study, expression of *IL6* and *TNF* in the adipose depots was positively associated (moderate) with the expression of *CD14*. Because ATM are mainly responsible for the *IL6* and *TNF* production in adipose tissue (Bruun et al., 2005), the results of the present study might suggest a pro-inflammatory polarization of ATM similar to the ATM in the study of Contreras et al. (2015). However, we have to be careful with this interpretation because we did not measure the expression of anti-inflammatory adipokines (*IL10*).

The greater expression of *IL6* in the internal depots is suggestive for a pro-inflammatory nature of these depots. Based on the positive association between adipocyte size and the expression of *IL6* and *TNF*, we hypothesize that in overconditioned cows, *IL6* and *TNF* originating from adipose depots with large adipocytes, activate the liver to produce pro-inflammatory molecules, which may lead to a dysregulation of inflammatory processes (Loor et al., 2006; Loor et al., 2007). This hypothesis could not be confirmed in the study of Akbar et al. (2015), however the BCS of the ‘overconditioned’ animals in that study (5.0 on a 10 point scale) was not as extreme as the BCS of the overconditioned cows in the present study (5.0 on a 5 point scale). Tumor necrosis factor links inflammatory processes with negative health consequences. Expression of *TNF* is increased in adipose tissue of DA cows and fatty liver cows had greater serum *TNF* (Ohtsuka et al., 2001; Contreras et al., 2015). Daily subcutaneous injections of *TNF* induced hepatic accumulation of triglycerides (Bradford et al., 2009). However when administered as a continuous low dose infusion in the adipose tissue, there was no obvious infiltration of fat in the liver and an anti-inflammatory reaction (increased *IL10* production) was observed in the liver and the adipose tissue (Martel et al., 2014). As such, it can be questioned if the locally increased production of pro-inflammatory cytokines in adipose depots has a negative influence on metabolic and inflammatory pathways. More research is needed to determine the potential relationship between the expression of pro- and anti-inflammatory molecules in adipose tissue of overconditioned cows, the inflammatory profile of these cows and potential health consequences.

6. CONCLUSIONS

The present research demonstrates a positive association between adipocyte size and the expression of pro-inflammatory adipokines, leptin, IL6 and TNF. The greater expression of *CD14* in adipose depots with larger adipocytes might suggest an increased infiltration of ATM in adipose depots from overconditioned cows. The lack of change in expression of *ADIPOQ*, an anti-inflammatory adipokine, does not exclude changes in serum concentration of adiponectin.

7. ACKNOWLEDGMENTS

This research was funded by the Special Research Fund of Ghent University, grant number 01D28410. The authors wish to thank Carolien Rogiers for excellent technical assistance in the lab.

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CHAPTER 7.2

Relationship between serum adiponectin concentration, body condition score and peripheral tissue insulin response of dairy cows during the dry period

De Koster J., Urh C., Hostens M., Van den Broeck W., Sauerwein H. and Opsomer G. Relationship between serum adiponectin concentration, body condition score and peripheral tissue insulin response of dairy cows during the dry period. 2016. Domestic Animal Endocrinology (Accepted).

1. ABSTRACT

The aim of the present study was to describe the relationship between serum adiponectin concentration and peripheral tissue insulin response in dairy cows with a variable body condition score (BCS) during the dry period. Cows were selected at the beginning of the dry period based on BCS (BCS 2.5 – 3.5, $n = 4$; BCS 3.75 - 5, $n = 5$). Animals were followed from the beginning of the dry period by weekly blood sampling and assessment of BCS and backfat thickness. Weekly blood samples were analyzed for adiponectin concentration using a bovine specific ELISA. Hyperinsulinemic euglycemic clamp tests were performed at the end of the dry period to measure peripheral tissue insulin response. Insulin dose response curves were established for both glucose and fatty acid metabolism. Regression analysis revealed that the serum concentrations of adiponectin dropped at the end of the dry period ($P < 0.05$) and were negatively associated with BCS ($P < 0.05$). At the level of the glucose metabolism, serum concentrations of adiponectin were positively correlated with insulin responsiveness (reflecting the maximal effect of insulin; $r = 0.76$, $P < 0.05$), but not with insulin sensitivity (reflecting the insulin concentration needed to achieve halfmaximal effect; $r = -0.54$, $P > 0.05$). At the level of the fatty acid metabolism greater adiponectin concentrations were negatively correlated with NEFA levels during the HEC test reflecting the insulin responsiveness of the NEFA metabolism ($r = -0.61$, $P = 0.08$), whereas there was no association with the insulin sensitivity of the NEFA metabolism ($r = -0.16$, $P = 0.67$). In conclusion, serum concentrations of adiponectin were negatively associated with the BCS in dairy cows during the dry period, and positively associated with insulin responsiveness of the glucose and fatty acid metabolism.

2. INTRODUCTION

Adiponectin, a protein exclusively produced by adipocytes, exerts insulin sensitizing and anti-inflammatory properties in different tissues and is recognized to play an important role in the pathogenesis of the human metabolic syndrome (Kadowaki and Yamauchi, 2005; Yamauchi and Kadowaki, 2008). Obesity, in humans and rodents, is associated with decreased circulating concentrations of adiponectin and a downregulated expression of AdipoR1 and AdipoR2. Both factors contribute to a disruption of normal metabolic function of different tissues which eventually will lead to the development of insulin resistance, type 2 diabetes mellitus and

cardiovascular disease (Tsuchida et al., 2004; Yamauchi and Kadowaki, 2008; Brochu-Gaudreau et al., 2010). Overconditioned dairy cows are known to be more susceptible for metabolic and infectious disorders, which is known as the fat cow syndrome (Morrow, 1976; Roche et al., 2013). Similarly to the human metabolic syndrome, the adipose tissue is an important contributor in the development of the fat cow syndrome due to the excessive release of fatty acids (Roche et al., 2013; De Koster et al., 2016b). The adipose tissue of dairy cows is also capable of producing different adipokines, including adiponectin (Giesy et al., 2012; Mielenz et al., 2013; Singh et al., 2014). However, the metabolic role of adiponectin in dairy cows is not yet fully explored. Recent in vitro research demonstrated that adiponectin decreases tumor necrosis factor (TNF) production by bovine monocytes (Kabara et al., 2014), while it stimulates lipid oxidation in bovine hepatocytes (Chen et al., 2013).

In a previous paper, we described the relationship between the accumulation of body fat and the glucose and fatty acid metabolism of peripheral tissues in response to insulin. Overconditioned cows were shown to have a decreased insulin sensitivity (increased $EC_{50_{\text{glucose}}}$) and a decreased insulin responsiveness (decreased max_{glucose}) of the glucose metabolism while insulin action at the level of the fatty acid metabolism was not influenced by BCS (De Koster et al., 2015). The aim of the present study was to describe the relationship of the serum adiponectin concentration during the dry period with: a) BCS, b) adipose depot weight, and c) variables derived from the insulin dose response curves of the glucose and fatty acid metabolism for cows of variable BCS during the dry period.

3. MATERIALS AND METHODS

All experimental procedures were approved by the ethical committee of the Faculty of Veterinary Medicine (EC2010/149 - University Ghent, Belgium). The study design is described in detail by De Koster et al. (2015). Ten clinically healthy, pregnant Holstein Friesian dairy cows were selected at the beginning of the dry period based on BCS according to the scale of Edmonson et al. (1989). The upcoming parity number of the animals was: 2 (n = 4); 3 (n = 4); 4 (n = 1) and 5 (n = 1). Five animals were considered to have a normal BCS (BCS 2.5 - 3.5) and 5 animals were considered to be overconditioned (BCS 3.75 - 5). Cows were followed starting 2 months before the expected parturition date by weekly assessment of BCS,

backfat thickness and weekly blood sampling. In the 3rd week (-21 to -17 d) before the expected parturition date, cows were weighed and catheters (Cavafix Certo 338-14G, B. Braun, Instrulife, Oostkamp, Belgium) were placed in both jugular veins. The next day, the animals underwent a hyperinsulinemic euglycemic clamp (HEC) test. Water and hay were always available but corn silage was withheld from 12 h before until the end of the test. The HEC tests were performed as described in detail by De Koster et al. (2015). Briefly, after assessment of basal blood glucose concentration, 4 consecutive insulin infusions were administered at increasing doses of insulin 0.1, 0.5, 2 and, 5 mIU/kg per min (Actrapid 100 IU/mL, human recombinant insulin, Novo Nordisk, Bagsvaerd, Denmark). At regular time points, blood glucose concentration was determined using a hand-held glucometer (Precision Xceed, Abbott Diabetes Care, Verdifarm, Beringen-Paal, Belgium) and compared with the basal blood glucose concentration measured before the start of the HEC test. When the blood glucose concentration dropped, the speed of the concomitant glucose infusion (30% glucose, Eurovet, Verdifarm, Beringen-Paal, Belgium) was increased to keep the blood glucose concentration near basal levels. A steady state was reached when no or minor changes of the glucose infusion (CV < 10 %) were necessary to keep the blood glucose concentration constant and near basal levels for at least 30 min. During each steady state, the steady state glucose infusion rate (SSGIR), the steady state insulin concentration (SSIC) and the steady state NEFA concentration (SSNEFA) were calculated as the average GIR, the average insulin concentration and the average NEFA concentration during the steady state period. Within 2 h after collection, all blood samples were centrifuged for 20 min (2,460 x g, 7 °C) and stored at - 80 °C until analysis. Samples for NEFA and insulin determination were taken in gel-coated blood tubes (Vacutest, Novolab, Geraardsbergen, Belgium). Serum insulin concentrations were determined using a human specific insulin electrochemiluminiscent immunoassay (ECLIA, Roche, Basel, Switzerland), intra- and inter-assay CV were 1.1% and 6.0%, respectively. Serum NEFA concentrations were determined in a commercial laboratory (Mediclab, Aalst, Belgium) using an enzymatic endpoint method, intra- and inter-assay CV were 1.0 % and 1.1 %, respectively. Serum adiponectin concentrations were determined by an indirect, competitive bovine specific ELISA (Mielenz et al., 2013), intra- and inter-assay CV were 4.5 % and 5.6 %, respectively.

One wk after the HEC test (-13 to -10 d before expected parturition date), cows were euthanized at the Department of Morphology (Faculty of Veterinary Medicine, Ghent University, Belgium). The day before euthanasia, live BW of the cows was determined using a large animal floor scale (Bascules Robbe NV, Torhout, Belgium). Immediately after euthanasia, adipose tissue was dissected from the 4 major adipose depots (subcutaneous, abdominal, intrapelvic, and thoracal), weighed and subdivided as described in detail by De Koster et al. (2015). The subcutaneous adipose depot contains all the adipose tissue that is located subcutaneously at the back, the sternum, and, the tailbase (including the adipose tissue in the fossa ischiorectalis). The abdominal adipose depot contains all the adipose tissue that is located in the omentum majus, omentum minus, mesenterium, and, the perirenal and retroperitoneal adipose tissue. The intrapelvic adipose depot contains all the adipose tissue that is located in the pelvic cavity. The thoracal adipose depot contains all the adipose tissue that is located at the inside of the ribs, in the mediastinum, and, around the heart (including the adipose tissue in the coronary grooves). All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). One cow (BCS = 2.82) was excluded from final statistical analysis because steady state conditions during the HEC test were not reached in 2 of the 4 insulin infusion periods of the HEC test. Body condition score and BFT during the dry period were modeled using the PROC MIXED function with cow as random factor and time points during the dry period as repeated measurements within cow. Significant differences between time points were checked using the Bonferroni adjustment for multiple comparisons. For each individual cow, dose response curves were fitted for the glucose and NEFA metabolism using the PROC NLIN function as described in detail by De Koster et al. (2015). The shape of the dose response curves of the glucose metabolism is determined by 2 factors: \max_{glucose} being the maximal SSGIR at maximal SSIC (reflecting insulin responsiveness of the glucose metabolism) and $EC50_{\text{glucose}}$ being the insulin concentration needed to achieve the half maximal effect (reflecting insulin sensitivity of the glucose metabolism). The shape of the dose response curves of the NEFA metabolism is determined by 2 factors: \min_{NEFA} being the minimal NEFA level at maximal SSIC (reflecting insulin responsiveness of the NEFA metabolism) and $EC50_{\text{NEFA}}$ being the insulin concentration needed to achieve the half maximal effect (reflecting insulin sensitivity

of the NEFA metabolism). Descriptive statistics (PROC MEANS) are expressed as mean \pm SD unless otherwise indicated. Normality of the variables were checked using the Kolmogorov-Smirnov test (PROC UNIVARIATE). Non-normally distributed variables were log transformed and checked for normal distribution using the Kolmogorov-Smirnov test. Assumption of normal distribution was met for every log transformed variable. A mixed model was built with adiponectin as dependent variable, cow as random factor, time point during the dry period as repeated measure within cow and BCS as independent variable (PROC MIXED). The interaction between BCS and time point was not significant and left out of the model. Comparison of the difference in adiponectin concentration between the last time point and the other time points was determined using the LSMESTIMATE statement. Pearson correlation coefficients between the different variables were calculated using the PROC CORR function. Significance and tendency were declared at $P < 0.05$ and $0.05 < P < 0.10$, respectively.

4. RESULTS

The comparison of BCS and BFT at different time points within individuals, revealed no significant difference, meaning that the BCS and BFT did not change during the dry period. Body condition score was negatively associated with adiponectin concentration (regression coefficient of the mixed model = - 3.54; $P < 0.05$). Time had a significant influence on the adiponectin concentration ($P < 0.05$) and we observed a drop in adiponectin concentration towards the end of the dry period independent of the BCS of the animals (Figure 1). The adiponectin concentration at the last sampling time point was different compared with the adiponectin concentration measured at the other time points ($P < 0.05$). Bodyweight of the animals was not associated with the adiponectin concentration at the moment of euthanasia. Similarly, no associations were found with other variables describing adiposity of the animals (Table 1).

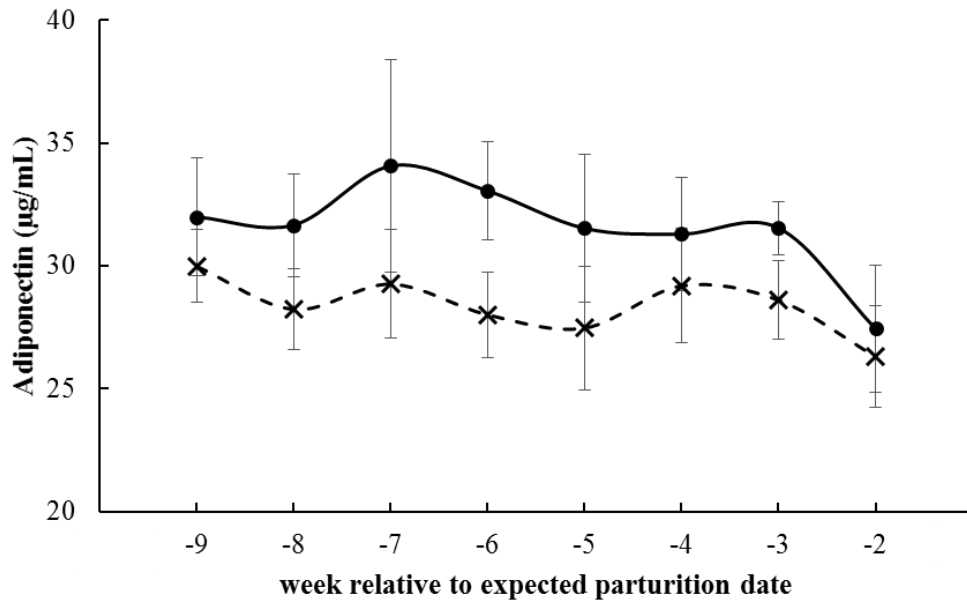


Figure 1. Serum concentration of adiponectin ($\mu\text{g/mL}$) during the dry period. Points represent the average adiponectin concentration of the animals at that time point, error bars represent the SEM. Dots (●) represent cows with $\text{BCS} < 3.5$ ($n = 4$); crosses (x) represent cows with $\text{BCS} > 3.75$ ($n = 5$).

Table 1. Body weight, backfat thickness, abdominal fat, subcutaneous fat, intrapelvic fat, thoracal fat and total fat. Pearson correlation coefficients (r) calculated between the serum adiponectin concentration at the moment of euthanasia and the individual variables ($n = 9$).

Item	Mean \pm SD	Serum adiponectin	
		r	P value
Body weight (kg)	724 ± 78	- 0.22	0.57
Backfat thickness (cm)	2.39 ± 1.15	- 0.06	0.87
Abdominal fat ^a (kg)	38.98 ± 21.47	- 0.10	0.80
Subcutaneous fat ^a (kg)	10.55 ± 6.45	- 0.14	0.71
Intrapelvic fat ^a (kg)	3.47 ± 1.69	- 0.19	0.62
Thoracal fat ^a (kg)	5.01 ± 2.49	- 0.09	0.82
Total fat ^a (kg)	58.01 ± 31.24	- 0.10	0.79

^a Variables were log transformed before the calculation of Pearson correlation coefficients.

No association could be demonstrated between adiponectin concentration at the moment of the HEC test and insulin sensitivity of the glucose ($\log(\text{EC}_{50_{\text{glucose}}})$, Figure 2A, $r = -0.54$, $P = 0.13$) and NEFA metabolism ($\log(\text{EC}_{50_{\text{NEFA}}})$, Figure 2C, $r = -0.16$, $P = 0.67$). Greater adiponectin concentrations at the moment of the HEC test were associated with a greater maximal stimulatory effect ($\text{max}_{\text{glucose}}$) of insulin on the glucose metabolism (Figure 2B, $r = 0.76$, $P < 0.05$). At the level of the NEFA metabolism, there was a trend for greater adiponectin concentrations to be associated with lower minimal NEFA levels (min_{NEFA}) ($r = -0.61$, $P =$

0.08, Figure 2D). The \min_{NEFA} reflects the insulin responsiveness of the NEFA metabolism, lower \min_{NEFA} values are indicative for a greater maximal inhibitory effect of insulin on the NEFA metabolism and thus a higher insulin responsiveness or lower insulin resistance.

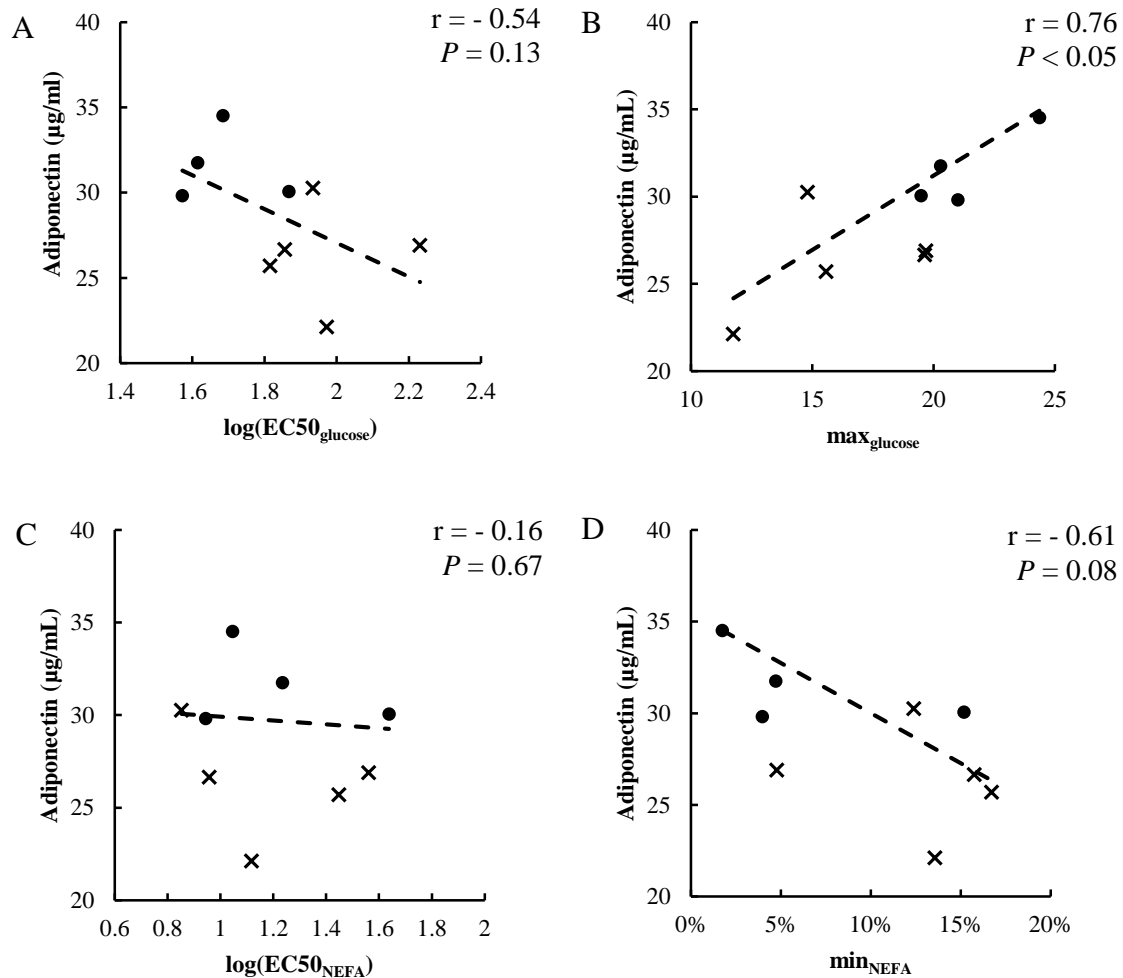


Figure 2. Relationship between the serum concentration of adiponectin (µg/mL) at the moment of the hyperinsulinemic euglycemic clamp test and $\log(\text{EC50}_{\text{glucose}})$ (A), \max_{glucose} (B), $\log(\text{EC50}_{\text{NEFA}})$ (C) and \min_{NEFA} (D). Dots (•) represent the cows with BCS < 3.5 (n = 4); crosses (x) represent cows with BCS > 3.75 (n = 5). The insulin sensitivity of the glucose and NEFA metabolism is reflected by $\text{EC50}_{\text{glucose}}$ and $\text{EC50}_{\text{NEFA}}$, respectively. The maximal effect of insulin on the glucose and NEFA metabolism (insulin responsiveness) is reflected by \max_{glucose} and \min_{NEFA} .

5. DISCUSSION

The medium and high molecular weight forms of adiponectin are the most important isoforms circulating in the blood of dairy cows (Giesy et al., 2012; Singh et al., 2014). In the present study, as previously demonstrated by others (Giesy et al., 2012; Mielenz et al., 2013), serum adiponectin concentration decreased at the end of the dry period. Since adiponectin exerts insulin sensitizing effects on the liver, skeletal muscle and, adipose tissue, low adiponectin

concentrations might contribute to the homeorhetic adaptation that supports the transition from pregnancy to lactation. In view of prioritizing glucose uptake by the mammary gland, low adiponectin concentrations around parturition are favorable because this would increase gluconeogenesis, increase lipolysis and decrease glucose uptake by peripheral tissues (Singh et al., 2014; Sauerwein and Häußler, 2015). The decreased adiponectin concentrations towards parturition might be regulated by increased concentrations of prolactin, growth hormone and, glucocorticoids at that time (Brochu-Gaudreau et al., 2010).

Excessive accumulation of body fat was associated with a decreased concentration of adiponectin in dairy cows during the dry period. Body condition score was negatively associated with the serum adiponectin concentration during the dry period. Contrary to the negative correlations between the serum concentrations and the adipose tissue mass demonstrated by Singh et al. (2014), the serum concentration of adiponectin in the present study was not associated with the amount of fat in the adipose depots at the moment of euthanasia. The discrepancy might be explained by the fact that, in the present study, animals were euthanized at the end of the dry period, when adiponectin concentrations started to decline. We hypothesize that at that moment, adiponectin concentrations are regulated by homeorhetic mechanisms rather than by body fatness. Although more research is needed to confirm our hypothesis. Treatment of bovine hepatocytes with adiponectin resulted in decreased triglyceride content due to the activation of both the adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor α (PPAR α) pathways that will stimulate fatty acid oxidation in hepatocytes (Chen et al., 2013). Decreased levels of adiponectin at the end of the dry period could decrease fatty acid oxidation, consequently, fatty acids will be redirected to other metabolic pathways (like triglyceride formation) (Lor et al., 2006; Brochu-Gaudreau et al., 2010). The decreased fatty acid oxidation by the liver in combination with the increased mobilization of NEFA by the adipose tissue observed in overconditioned dairy cows (De Koster et al., 2016b) will increase the risk for triglyceride accumulation in the liver and hence hepatic lipidosis might occur.

Previous studies found a weak association between serum adiponectin concentration and RQUICKI as measure of insulin sensitivity (Singh et al., 2014). The use of surrogate indices

to assess insulin sensitivity is however questionable in dairy cows, especially in the periparturient period when dramatic changes of the individual variables (glucose, insulin and, NEFA) take place (De Koster et al., 2016a). The gold standard method to measure insulin resistance is the HEC test, and by using incremental insulin doses, it is possible to assess both insulin sensitivity and insulin responsiveness (Kahn, 1978; De Koster and Opsomer, 2013). In the present study, we demonstrated a positive association between the average adiponectin concentration and insulin responsiveness of both the glucose and the NEFA metabolism of cows during the dry period but not insulin sensitivity. Insulin responsiveness reflects the maximal effect of insulin on the glucose and NEFA metabolism at high insulin concentrations, while insulin sensitivity reflects the insulin concentration needed to achieve the halfmaximal effect. Whether this observation has biological relevance is questionable because insulin responsiveness of the glucose and NEFA metabolism was, in the present study, measured at insulin concentrations well above physiological concentrations during the dry period (De Koster et al., 2015). A recent study (Weber et al., 2016) demonstrated no difference in adiponectin concentration or peripheral tissue insulin response as measured by the HEC test between cows differing in liver fat accumulation. The difference with our study can be explained by the fact that the difference in BCS of the animals in the present study was much greater and the fact that in the present study, four different insulin infusions were used while in the study of Weber et al. (2016), only one insulin dose (6 mU/kg BW per min) was used.

6. CONCLUSIONS

Serum adiponectin concentration in the dry period was negatively associated with the BCS of dairy cows during the dry period. Independently of the BCS of the animals, the serum adiponectin concentration decreased at the end of the dry period. In the present study, serum adiponectin concentrations were positively associated with the insulin responsiveness of the glucose and NEFA metabolism. More research is needed towards the functional role of adiponectin in peripheral tissue metabolism of periparturient dairy cows and the development of transition problems.

7. ACKNOWLEDGMENTS

This research was funded by the Special Research Fund of Ghent University, grant number 01D28410. The authors wish to thank Lars Hulpio, Isabel Lemahieu and Petra Van Damme for excellent technical assistance in the lab.

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CHAPTER 8

General discussion

The aim of this doctoral thesis was to identify key factors in the pathophysiology of the increased disease susceptibility in overconditioned dairy cows during the transition period. Central hypothesis was that excessive insulin resistance and excessive fat accumulation play an important role in the derailment of normal metabolic function of multiple tissues at the end of pregnancy. However, we encountered the problem that assessment of insulin resistance in dairy cows is not straightforward. Therefore, we first validated and evaluated different methodologies to assess insulin resistance in dairy cows. In a next experiment, dairy cows at the end of the dry period were carefully selected based on body condition score (BCS) and enrolled in a study to determine the relationship between overconditioning, insulin resistance and metabolic as well as pro-inflammatory properties of the adipose tissue. In the present Chapter 8, we will provide more insight in the practical relevance and implications of our results and propose some opportunities for further research.

1. HOW TO MEASURE INSULIN RESISTANCE IN DAIRY COWS?

1.1. General concept of insulin resistance in dairy cows

In the periparturient period, dairy cows limit peripheral glucose use to maximally preserve glucose for the pregnant uterus or the lactating mammary gland. The latter is in the first place achieved by lowering circulating concentrations of insulin (Bell and Bauman, 1997; Vernon and Pond, 1997; Bauman, 2000). Additionally, it is generally accepted that dairy cows go through a transient period of insulin resistance of the glucose metabolism at the end of pregnancy and the beginning of lactation (Bell, 1995; Bell and Bauman, 1997; Vernon and Pond, 1997). However, up till now, direct evidence of this insulin resistant state in otherwise healthy dairy cows is rather limited. Most of the initial research towards insulin resistance as part of the homeorhetic adaptation to early lactation has been done in sheep and goats:

- in goats, HEC tests revealed decreased insulin responsiveness (at maximal insulin concentrations) of the whole body insulin stimulated glucose uptake in early lactating goats versus goats in late lactation and the dry period (Debras et al., 1989);
- lactation was characterized by a lower disappearance of glucose through the hindlimb of sheep during a HEC test (Vernon et al., 1990);

- sheep at the end of pregnancy demonstrated reduced insulin sensitivity of the whole-body insulin stimulated glucose uptake in comparison with nonpregnant-nonlactating sheep (Pettersson et al., 1993);
- in adipose tissue of sheep in early lactation, insulin stimulated glucose uptake was decreased compared with nonlactating-nonpregnant sheep (Vernon and Taylor, 1988).

Similarly, in humans and rodents, insulin resistance of the glucose metabolism develops in the second half of pregnancy and progresses until the end of pregnancy. Pregnancy induced insulin resistance is thought to be mediated by pregnancy associated hormones: progesterone, cortisol, prolactin, human placental lactogen and human placental growth hormone (Stanley et al., 1998; Barbour et al., 2007). However, normal insulin sensitivity is restored after delivery in humans (Stanley et al., 1998; Barbour et al., 2007), in contrast to what has been observed in sheep, goats and dairy cows. One can question if insulin resistance during lactation is a typical phenomenon for ruminants or biased by the specific glucose metabolism of ruminants and the way we measure insulin sensitivity in ruminants, or maybe due to the intense genetic selection towards milk production in the dairy cow?

After all these years of research, still questions remain to be answered, such as whether dairy cows are insulin resistant in the periparturient period, whether there is variation in the level of insulin resistance between cows and whether this insulin resistant state has an impact on the health of individual cows. Answering these questions would ideally involve a longitudinal study where cows are enrolled during the dry period and peripheral tissue insulin response is measured repeatedly throughout the dry period and lactation. Choosing the most appropriate method to measure the peripheral tissue insulin response in this follow-up experiment is the next challenge. The following methods have been suggested to assess insulin resistance in dairy cows (De Koster and Opsomer, 2013):

- the hyperinsulinemic euglycemic clamp test;
- the intravenous glucose tolerance test;
- the intravenous insulin tolerance test;
- the surrogate indices for insulin sensitivity like QUICKI, RQUICKI, RQUICKI_{BHB};

- biopsies of skeletal muscle, adipose tissue or liver after insulin or glucose challenge and measuring activation of selective proteins;
- in vitro experiments with skeletal muscle, adipose tissue or liver.

The method of choice depends on the purpose of the experiment. Generally, in dairy cows, it is very challenging to accurately assess insulin resistance or insulin sensitivity of the glucose metabolism due to the fact that the glucose metabolism is largely regulated in an insulin independent way (De Koster and Opsomer, 2013).

1.2. Comparing cows in the same physiological state

Cows in the same physiological state (the same stage of pregnancy or the same amount of milk production in a similar stage of lactation), are expected to have a similar amount of glucose disappearing independently of insulin to the gravid uterus or the lactating mammary gland. In Chapter 4.1, we demonstrated that surrogate indices for insulin sensitivity are not useful to compare insulin sensitivity in cows at the end of pregnancy. Compared to the gold standard method to assess insulin resistance of the glucose metabolism (the HEC test), the AUC of glucose derived from a GTT is a reliable parameter to assess insulin resistance. A drawback of the AUC is that it does not take into account differences in insulin secretion during the GTT. Therefore, the MINMOD analysis of a GTT, is expected to be more reliable to assess insulin resistance due to the fact that the minimal model accounts for differences in insulin secretion between animals. The derived variable is the insulin sensitivity index S_i , which reflects the ability of insulin to enhance glucose disappearance during a GTT. The results from the study in Chapter 4.1 are related to cows at the end of pregnancy and more research is needed to validate different measures of insulin sensitivity of the glucose metabolism in lactating cows. Similarly to our results, Schoenberg et al. (2012) concluded that results derived from HEC tests and GTT correspond with each other, whereas RQUICKI was unable to confirm results derived from the HEC test and GTT. Kerestes et al. (2009) and Mann et al. (2016c) found no correlations between surrogate indices for insulin sensitivity and insulin sensitivity measures derived from GTT and ITT. Therefore, the use of surrogate indices to assess insulin resistance in dairy cows should be confined to those physiological conditions where it has been clearly proven that they can be used. Unfortunately, until now, none of the surrogate indices have been

proven to be reliable in dairy cows. In the present thesis, we did not perform any ITT but based on results from McCann and Reimers (1985), Oikawa and Oetzel (2006), Pires et al. (2007), Kerestes et al. (2009), we suggest that ITT can be useful to compare peripheral tissue response between cows in a similar physiological state.

1.3. Comparing cows in a different physiological state

As described in Chapter 4.2, pregnancy and lactation have an important influence on whole body glucose metabolism. The insulin independent glucose disappearance is increased in animals at the end of pregnancy (where 60 to 70% of the glucose is shifted to the pregnant uterus) and this is even more pronounced in lactating animals (where 50 to 82% of the glucose is shifted to the lactating mammary gland) (Bickerstaffe et al., 1974; Bell and Bauman, 1997; Rose et al., 1997; Overton, 1998; Bell et al., 2000; De Koster and Opsomer, 2013). Petterson et al. (1993) described that pregnant sheep had higher insulin independent glucose utilization compared with nonpregnant sheep in the basal state. Due to the large variation in insulin independent glucose disappearance in the different stages of lactation, it is difficult to compare the insulin sensitivity of the glucose metabolism between cows in different physiological states (De Koster and Opsomer, 2013). It is impossible to correct for differences in insulin independent glucose disappearance using results from GTT or HEC tests as has been performed in the present thesis. To correctly account for differences in insulin independent glucose disappearance between cows in different physiological states, one needs to perform HEC tests in combination with an infusion of radioactive or stable isotopes of glucose as described by Petterson et al. (1993) in sheep and by Rose et al. (1997) and Weber et al. (2016) in dairy cows. The use of radiolabeled or stable isotopes of glucose makes the HEC test an even more labor intensive and expensive method limiting the number of animals that can be enrolled in the study (Muniyappa et al., 2008). Other methods might be more suitable to assess insulin resistance in dairy cows and this is discussed further below.

1.4. Insulin resistance of different metabolic pathways

As we have shown in Chapter 5, an insulin resistant state of the glucose metabolism does not necessarily entail an insulin resistant state of the NEFA metabolism. Similarly, Vernon (2005b) stated that different metabolic pathways are characterized by differences in insulin sensitivity.

As mentioned above, some metabolic responses to insulin are attenuated in the periparturient period in ruminants, such as whole body insulin stimulated glucose uptake (Debras et al., 1989; Petterson et al., 1993), stimulation of skeletal muscle glucose uptake (Vernon et al., 1990), stimulation of adipose tissue glucose uptake (Vernon and Taylor, 1988), stimulation of lipogenesis (Vernon, 2005b) and inhibition of hepatic gluconeogenesis (Bauman, 2000). In contrast, the insulin response of other metabolic pathways seems to be preserved or even enhanced in the periparturient period in ruminants: antilipolytic effect of insulin (Vernon, 2005b) and insulin's inhibitory effect on proteolysis (Tesseraud et al., 2007). The difference in insulin sensitivity of different metabolic pathways implicates that one needs to be careful when extrapolating results based on one metabolic pathway to another.

1.5. Most ideal method to measure insulin resistance in dairy cows

Insulin resistance is defined by Kahn et al. (1978) as a decreased biological response of insulin sensitive tissues on a certain amount of insulin. Accordingly, the most ideal method to measure insulin resistance in dairy cows would be a method where the administered amount of insulin is standardized and the biological response or metabolic pathway of interest is clearly identified and measured.

Standardization of the insulin dose is necessary because it is impossible to correct for the whole body insulin response of cows exposed to different insulin levels. This standardization is achieved during the HEC test and the ITT. Glucose tolerance tests are not suited because the largest variation observed between cows during GTT is at the level of the glucose induced insulin secretion (Chapter 4.1 and 4.2). The large variation in insulin secretion is undesirable in view of assessing insulin resistance. On the other hand, this is very interesting, because this implies that the reaction of the pancreas on a certain amount of glucose varies considerably between cows. If there is variation in a variable, there have to be factors explaining this variation and there will be consequences of this variation. So, more research is warranted to identify the causes and consequences of this variation in insulin secretion following a standardized glucose bolus. During HEC tests and ITT, a certain dose of insulin is administered continuously or via a bolus injection, respectively. During ITT, glucose concentrations drop considerably and counter regulatory mechanisms (glycogenolysis, lipolysis) are activated to

compensate for the hypoglycemia. So, measures of insulin sensitivity derived from ITT might be confounded by variation of these counter regulatory mechanisms between cows. Based on glucose and NEFA concentrations measured during ITT, one needs to calculate the proportional decrease compared to the basal glucose concentration to correct for differences in basal glucose and NEFA concentration between animals, as has been described by Pires et al. (2007) and Kerestes et al. (2009).

The HEC test is superior compared to the ITT due to the fact that the blood glucose concentration is kept at basal levels. Ideally, to measure the insulin sensitivity of the glucose metabolism, one should also assess basal glucose disappearance during the HEC test by infusing stable or radioactive isotopes of glucose as has been done by Rose et al. (1997) and Weber et al. (2016). As such, one can compare cows in different physiological states with different basal glucose disappearance. Stable isotopes (^{13}C glucose or ^2H glucose) do not pose the health risks of radioactive isotopes and are preferred to be used in these studies (Drackley et al., 2006). Hyperinsulinemic euglycemic clamp tests based on multiple insulin infusions are superior compared to HEC tests with a single insulin infusion due to the fact that one can easily model dose response curves using non-linear regression analysis. The dose response curves for insulin are sigmoidal and can be described by Michaelis-Menten kinetics (Brehm and Roden, 2007). From the equations of the dose response curves, two meaningful physiological parameters are distilled: the maximal effect of insulin (max) and the insulin concentration needed to achieve the half maximal effect (EC50) (Brehm and Roden, 2007). While EC50 determines the insulin sensitivity of a biological process, the maximal effect of insulin determines the insulin responsiveness of a biological process (Kahn, 1978). A drawback of the HEC test is that it measures metabolism at supraphysiological insulin concentrations which might limit the biological relevance of the results (Schoenberg et al., 2012).

Another suitable way of assessing insulin sensitivity of different metabolic pathways would be to measure tissue-specific insulin signaling as has been done by Ji et al. (2012), Zachut et al. (2013) and Mann et al. (2016a; 2016b). In the study of Ji et al. (2012), adipose tissue samples were taken from the tail head region of the cows and incubated in vitro with or without insulin. In the study of Zachut et al. (2013) and Mann et al. (2016a; 2016b) biopsies of the tissue of

interest (liver, skeletal muscle or adipose tissue) were taken before and after administration of a glucose bolus. After sampling of tissues *in vitro* or *in vivo*, the activation of the intracellular insulin signaling cascade was measured by western blot (Ji et al., 2012; Zachut et al., 2013; Mann et al., 2016a; Mann et al., 2016b). By comparing the activation (phosphorylation) of different proteins responsible for the intracellular signal transduction (Akt, HSL, Erk, ...) in the basal and stimulated state, it is possible to get an idea about the activation and sensitivity of that specific pathway in that specific tissue by insulin. Metabolic pathways of insulin are mainly controlled by Akt/PI3 kinase while mitogenic pathways of insulin are mainly controlled by MAPK/ERK (Figure 1) (Sasaki, 2002; Vernon, 2005b; Baumgard et al., 2016).

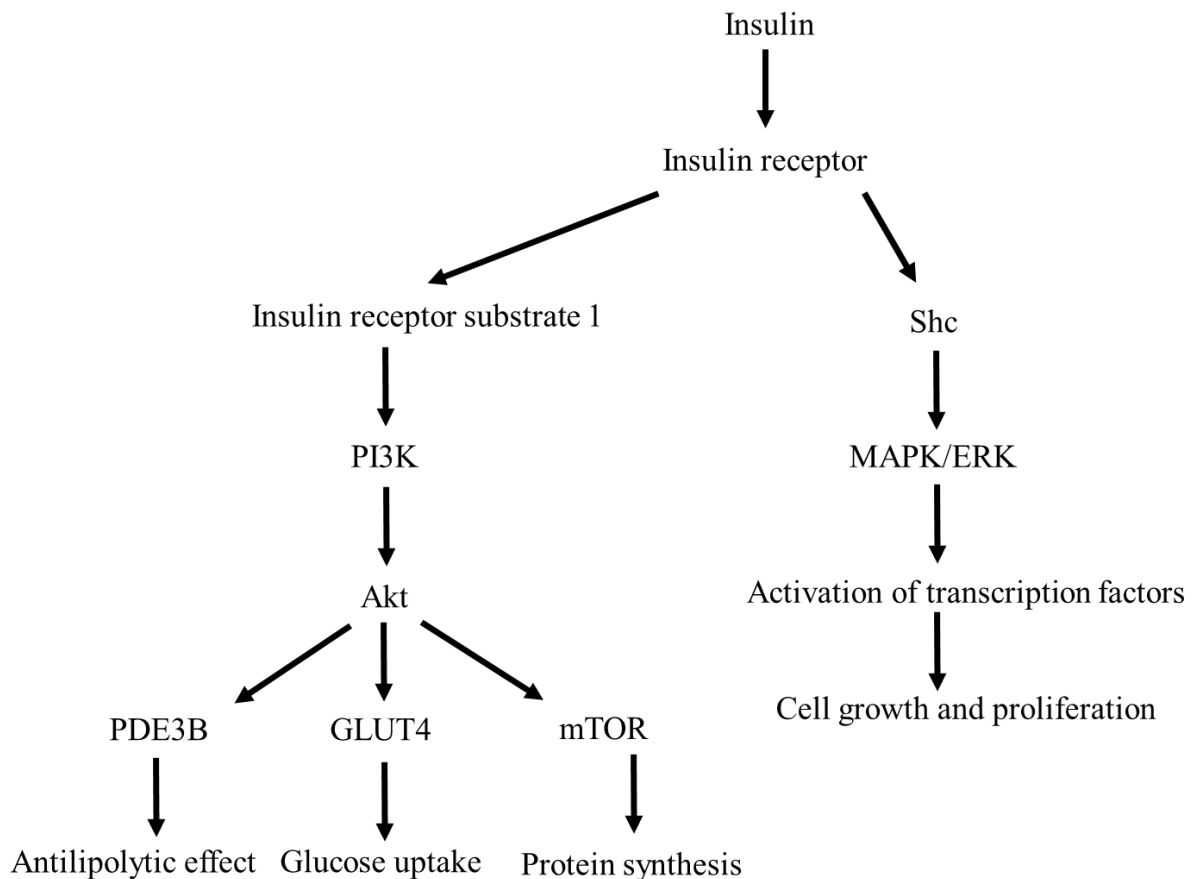


Figure 1: Intracellular insulin signaling cascade divided by metabolic pathways (left) and mitogenic pathways (right). PI3K, phosphatidylinositol 3-kinase; Akt, Protein kinase B; Shc, src homology and collagen related protein; MAPK, mitogen activated protein kinase; ERK, extracellular regulating protein kinase; PDE3B, phosphodiesterase 3B; GLUT4, glucose transporter 4; mTOR, mammalian target of rapamycin. Adapted from Vernon and Pond, 1997; Sasaki, 2002, Tesseraud et al., 2007.

A drawback is that western blots are semi-quantitative and possibly not sensitive enough to detect small differences in activation or phosphorylation of specific proteins (Mann et al., 2016a). Other drawbacks are that for most of the proteins no bovine-specific antibodies can be found and the method is invasive as one needs to take biopsies. In the study of Zachut et al., (2013) and Mann et al. (2016a; 2016b) activation of the insulin signaling cascade (as measured by phosphorylation of Akt) in liver, adipose tissue and skeletal muscle, by means of glucose induced insulin secretion following an intravenous glucose infusion, was lower in postpartum cows as compared with prepartum cows. However, glucose induced insulin secretion following the intravenous glucose bolus was lower in the postpartum period. This difference in secretion and thus exposure to insulin precludes direct conclusions towards insulin resistance of the metabolic pathways. The lower insulin secretion after a glucose bolus in the postpartum period is a typical observation and contributes to the homeorhetic adaptation mechanisms supporting lactation (Bossaert et al., 2008; Weber et al., 2016). Therefore, those studies investigating tissue-specific insulin signaling can be optimized by using one or more standardized insulin boluses instead of a glucose bolus.

2. THE FAT COW SYNDROME REVISITED

The immediate postpartum period of dairy cows is characterized by an increased glucose output via the lactating mammary gland. It was estimated that dairy cows lack approximately 1,000 g glucose per day in the beginning of lactation to keep glucose at the preferred physiological level (Lucy et al., 2013). Due to the lower physiological glucose concentration in early lactation and as part of the homeorhesis supporting lactation, different metabolic pathways will be activated to sustain the glucose requirements of the lactating mammary gland. Hepatic gluconeogenesis is upregulated and glucose use by peripheral tissues is downregulated. To fulfil total body energetic requirements, muscle and adipose tissue will start to breakdown to supply amino acids and glycerol for gluconeogenesis and NEFA and ketone bodies will be mobilized as alternative energy source for different tissues (Ingvarsen, 2006). To stay healthy and productive, dairy cows have to find a balance between glucose provision for milk production and adipose tissue breakdown. Approximately 30 to 50 % of all dairy cows develop at least one disorder in the periparturient period (Leblanc, 2010; Hostens et al., 2012a).

Especially excessive lipid mobilization is an important risk factor in the derailment of normal metabolism and the development of different transition disorders (Drackley, 1999).

Fat or overconditioned cows are more susceptible for the derailment of the metabolic function of different organs in the periparturient period. Different factors contribute to this increased disease susceptibility. In the first place, fat cows have a lower dry matter intake resulting in a more severe negative energy balance in the periparturient period (Rukkwamsuk et al., 1999; Grummer et al., 2004). Secondly, it was hypothesized that overconditioned cows suffer from excessive insulin resistance of the peripheral tissues which might provoke an increase in lipolysis (Holtenius et al., 2003; Pires et al., 2007). In the third place, overconditioned cows will mobilize more fat and are therefore more prone for fatty liver and ketosis (Vernon, 2005a; Roche et al., 2009; Roche et al., 2013). Although overconditioned cows suffer from higher levels of NEFA in the periparturient period (Pires et al., 2013), the reason of this observation was never clearly established. It was hypothesized that the following factors could contribute: an enhanced lipolytic activity of adipocytes, insulin resistance at the level of the adipocytes, a greater amount of fat available to mobilize or a combination of these factors (Rukkwamsuk et al., 1998; Herdt, 2000; Kokkonen et al., 2005). Furthermore, it is proposed that a dysregulated production or expression of adipokines in the adipose depots of overconditioned cows might evoke a pro-inflammatory state with a detrimental impact on normal functioning of different cells and organs. The role of insulin resistance, adipose tissue lipolysis and the pro-inflammatory state on the metabolism of overconditioned cows in the transition period, will be discussed further below.

2.1. Insulin resistance in overconditioned cows

Insulin resistance was speculated to play an important role in the derailment of the metabolism of overconditioned dairy cows in the periparturient period based on 3 observations:

- overconditioned cows have elevated NEFA levels in the periparturient period (Lacetera et al., 2005; Pires et al., 2013);
- glucose clearance after an insulin bolus was less pronounced in overconditioned compared with normally conditioned heifers indicative for an insulin resistant state in overconditioned heifers (McCann and Reimers, 1985);

- artificial elevation of NEFA and/or triacylglycerol in the blood of dairy cows was associated with the development of insulin resistance (Oikawa and Oetzel, 2006; Pires et al., 2007).

The following vicious circle was hypothesized: overconditioned cows are exposed to higher levels of NEFA and these NEFA play a causative role in the development of insulin resistance which in turn would provoke an increase in circulating NEFA due to an impaired inhibitory effect of insulin on the lipolytic pathway (Figure 2A) (Holtenius et al., 2003; Pires et al., 2007; Salin et al., 2012).

While the link between overconditioning and elevated periparturient NEFA levels is well established and confirmed in the present thesis, several steps of this vicious circle were not proven:

- insulin resistance was only demonstrated in overconditioned heifers (McCann and Reimers, 1985) that were not pregnant and not lactating, which limits the extrapolation towards dairy cows at the end of pregnancy or at the beginning of lactation;
- insulin resistance (McCann and Reimers, 1985; Oikawa and Oetzel, 2006; Pires et al., 2007) was only demonstrated at the level of the glucose metabolism, and not at the level of the NEFA metabolism.

To elucidate this vicious circle, we performed HEC tests in 10 cows carefully selected based on BCS to clarify the relationship between BCS and insulin response of the glucose and NEFA metabolism (Chapter 5). We demonstrated a negative association between the accumulation of body fat and the insulin sensitivity and insulin responsiveness of the glucose metabolism in dairy cows at the end of pregnancy. At the level of the NEFA metabolism, overconditioned cows reacted in a similar way on insulin compared with normal conditioned cows. It was concluded that overconditioning during the dry period is associated with an insulin resistant state of the glucose metabolism but not of the NEFA metabolism.

Therefore the link between insulin resistance and increased lipolysis is not retained. It was suggested that the NEFA lowering effect of insulin is preserved in overconditioned cows at the end of pregnancy. This is favorable because if cows maintain sufficient insulin levels in the periparturient period, insulin remains effective in inhibiting lipolysis. Nevertheless, a causative

role for NEFA may be expected in the development of insulin resistance of the glucose metabolism, as described in Chapter 5 (Figure 2B).

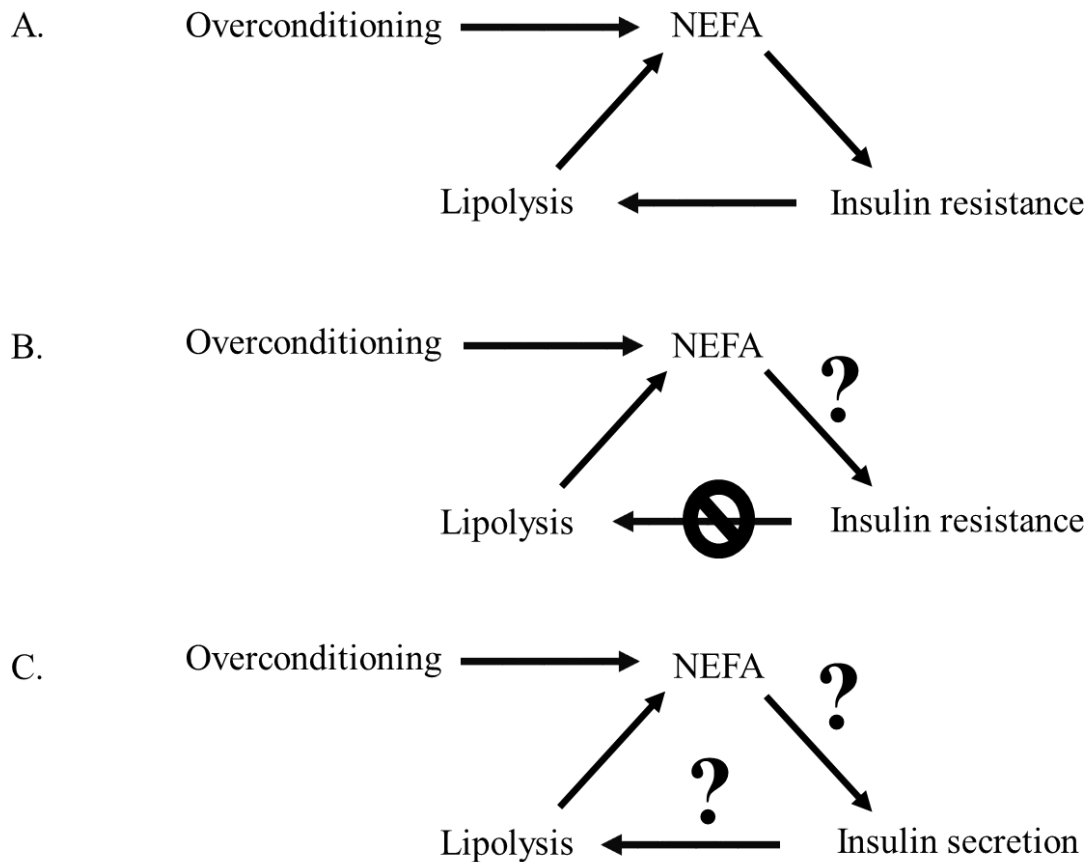


Figure 2: Proposed vicious circle of the relationship between NEFA, insulin resistance and lipolysis in overconditioned dairy cows (A). Insulin resistance was not established at the level of the NEFA metabolism but high levels of NEFA might be causative for an increased insulin resistant state of the glucose metabolism (B). An alternative hypothesis would be that high NEFA levels in overconditioned cows would limit insulin secretion by the pancreas and favor lipolysis (C).

More research is needed to confirm whether the decrease in insulin sensitivity and insulin responsiveness associated with overconditioning is also existent in the peri- and postpartum period. However, in case we can extrapolate the results from Chapter 5 to this period, we conclude that insulin resistance does not play a role in the excessive lipomobilization in overconditioned dairy cows. Other factors may be responsible for the excessive release of NEFA from the adipose tissue of overconditioned cows.

As demonstrated in Chapter 6, large adipocytes from overconditioned cows are characterized by an increased basal and stimulated lipolytic activity in vitro. We suggest that this might contribute to the elevated periparturient levels of NEFA in overconditioned cows.

Additionally, in humans, it is mentioned that chronic exposure to high levels of NEFA is associated with a reduced insulin secretion by the pancreas due to a lipotoxic effect of NEFA on the β cells of the pancreatic islets (Jensen, 2006; Kahn et al., 2006). Similar associations between chronically elevated NEFA concentrations and insulin secretion by the pancreas have been observed in dairy cows (Bossaert et al., 2008). Therefore, we hypothesize that in cows chronically exposed to high NEFA (like overconditioned cows) a lower insulin secretion might favor lipolysis (Figure 2C). However, in the present thesis, there was no association between BCS and basal insulin concentration or insulin secretion after a glucose challenge. More research is needed to demonstrate a causal relationship between serum NEFA concentration, insulin secretion of the pancreas and excessive lipolysis of adipose tissue.

2.2. Insulin response of different metabolic processes

Another interesting finding was that the insulin sensitivity was higher for the NEFA metabolism compared with the glucose metabolism (Chapter 5). This means that at a certain concentration of insulin, the effect of insulin will be greater at the level of the NEFA metabolism compared to the glucose metabolism. This has been mentioned previously:

- *'Insulin's actions in lipid metabolism are powerful and occur at low blood concentrations.'* (Herdt, 2000);
- *'Curiously, whereas the ability of insulin to stimulate lipogenesis in adipose tissue is diminished during lactation, the antilipolytic effect of insulin is retained.'* (Vernon, 2002);
- *'The plasma NEFA response to insulin appears to be more sensitive than the stimulatory effect of insulin on whole-body glucose utilization and the inhibitory effect of insulin on glucose production.'* (Pethick et al., 2005);
- *'The ED50 for this antilipolytic response was generally lower than that for the reduction in endogenous glucose production or increase in peripheral glucose utilization observed in the same animals.'* (Pettersson et al., 1994).

The higher insulin sensitivity of the NEFA metabolism compared with the glucose metabolism is logical and can be visualized as depicted in Figure 3.

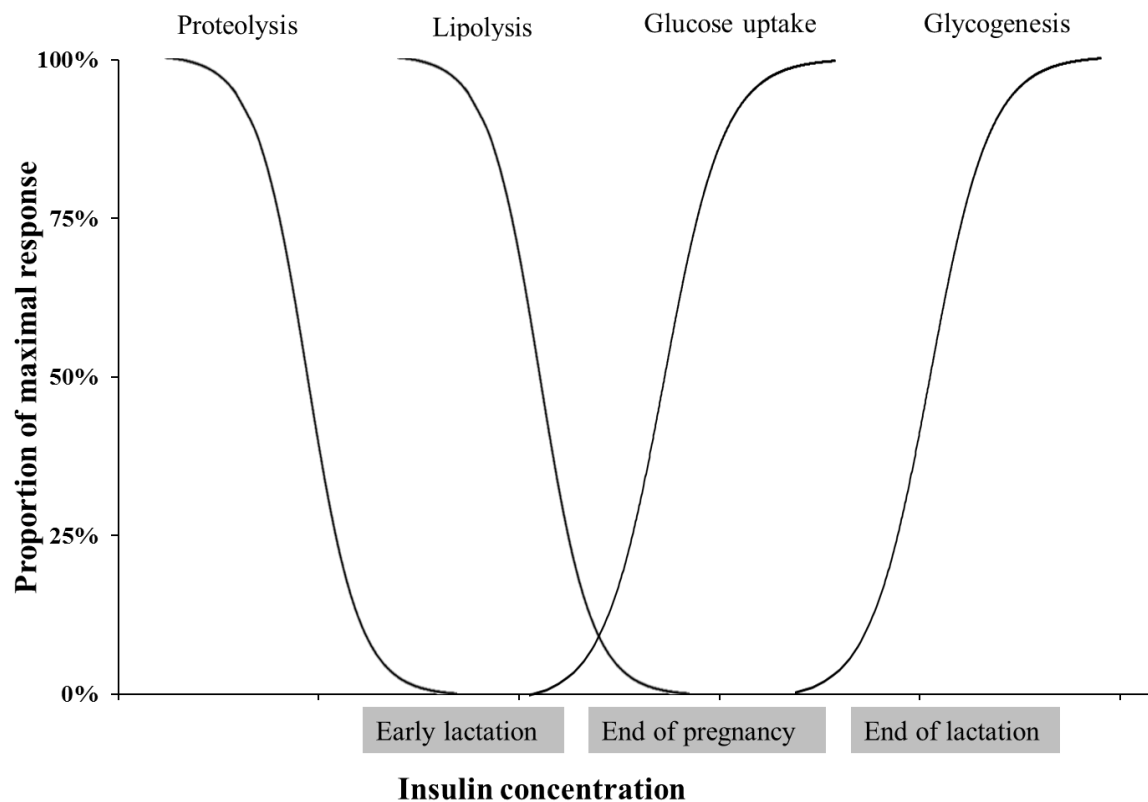


Figure 3: Effect of insulin on different metabolic processes during different stages of the lactation cycle (adapted from Vernon, 2005b).

During positive energy balance, like at the end of lactation (right bar of Figure 3), insulin levels are high (Herbein et al., 1985; Accorsi et al., 2005) and at that moment there is no proteolysis or lipolysis; glucose uptake by skeletal muscle, adipose tissue and other peripheral tissues is maximal and excess glucose is stored as triglycerides or glycogen. At the end of pregnancy (middle bar of Figure 3), insulin concentrations decrease (Herbein et al., 1985; Accorsi et al., 2005) and there will be a decrease in glucose uptake by skeletal muscle, adipose tissue and other peripheral tissues. In early lactation (left bar of Figure 3), insulin concentrations continue to decrease (Herbein et al., 1985; Accorsi et al., 2005) and very little glucose will be utilized by skeletal muscle, adipose tissue and other peripheral tissues, cows will start to mobilize adipose reserves and amino acids will be mobilized from skeletal muscle to alleviate negative energy balance.

2.3. Lipomobilization in overconditioned cows

2.3.1. Mass effect and differences in reactivity of adipocytes on hormonal stimuli

Excessive fat mobilization is an important risk factor for different periparturient disorders like ketosis and fatty liver (Drackley, 1999). Mobilization of NEFA occurs especially in the last days before calving and in the first days post partum (McCarthy et al., 2015a). In Chapter 5 and 6, we identified risk factors for excessive fat mobilization in dairy cows at the end of gestation. The first one is really simple, overconditioning is a risk factor for excessive mobilization of body fat. Not only because of the fact that overconditioned cows have a lower DMI (Grummer et al., 2004) but also because of the fact that overconditioned cows have more fat (mass effect) (Chapter 5). The more fat there is, the more NEFA will be released upon stimulation (Figure 4). Additionally, overconditioned cows have larger adipocytes. In vitro, these adipocytes will release more NEFA in the basal state and upon stimulation with catecholamines (Figure 4) (Chapter 6). Both factors, most probably, contribute to the fact that overconditioned cows will be exposed to higher levels of NEFA in the periparturient period. Similar to our in vivo observations, in vitro results failed to demonstrate an insulin resistant state of the antilipolytic effect of insulin in large adipocytes (Chapter 5 and 6).

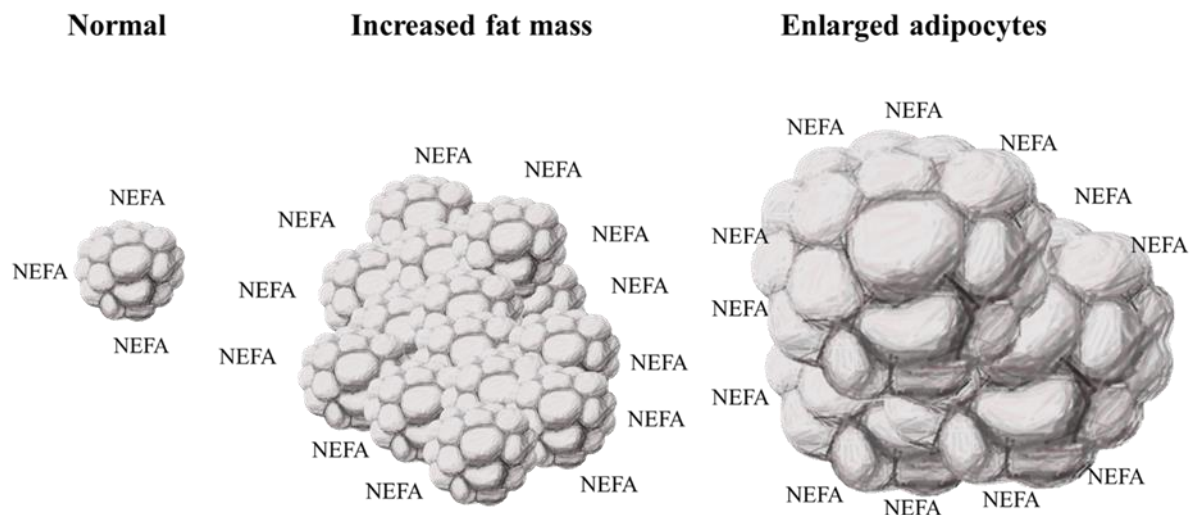


Figure 4: Comparison between the mass effect of adipose stores (increased fat mass) on NEFA release and the effect of adipocyte size (enlarged adipocytes) on NEFA release. Upon stimulation to release NEFA, animals with a greater adipose tissue mass, will release more NEFA. Upon stimulation to release NEFA, animals with larger adipocytes, will release more NEFA.

The mass effect is probably also the reason why the serum NEFA profile resembles more the NEFA profile of the abdominal adipose tissue compared with the subcutaneous adipose tissue (Contreras et al., 2010; Hostens et al., 2012b). The mass of the abdominal adipose tissue covers 60 to 70 % of the dissected adipose tissue mass while the subcutaneous adipose tissue accounts for only 10 to 20% herein (Chapter 5). Hence, when mobilization of adipose tissue starts, it is logical that the largest part of the circulating NEFA will originate from the abdominal adipose tissue. Despite differences in basal and stimulated lipolytic activity and insulin sensitivity between depots, the mass effect probably overrules the small differences in metabolic activity between depots

2.3.2. Comparison between overfeeding and overconditioning

Overfeeding of dairy cows during the dry period is another important risk factor linked to excessive fat mobilization in the periparturient period. It is known that cows overfed during the dry period, and cows overconditioned at the moment of parturition, show a remarkable increase of NEFA and BHBA in the first weeks post partum (Dann et al., 2006; Pires et al., 2013). Interestingly, the changes observed in the present study, in adipose tissue of overconditioned cows in the prepartum period, show some remarkable similarities with changes occurring in the adipose tissue of overfed cows in the postpartum period.

Overfed cows, like overconditioned cows, tend to have a higher maximal in vitro response to lipolytic stimuli when compared with normally fed cows (Rukkwamsuk et al., 1998). Ji et al. (2012; 2014) found that the expression of adipose triglyceride lipase, the main regulator of basal lipolytic activity, was upregulated in adipose tissue of overfed cows. And as in overconditioned cows in the present study, the insulin response of the adipose tissue was not significantly impaired in cows overfed during the dry period (Ji et al., 2012). We hypothesize that the similarities between overconditioned prepartum cows and overfed postpartum cows might be explained by an increased adipocyte size.

Chilliard et al. (2000) postulated that basal and catecholamine stimulated lipolytic activity are related to body fatness (adipocyte size) and energy balance. In the prepartum period, overfed dairy cows will increase lipogenic activity due to the increased availability of energy substrates (Rukkwamsuk et al., 1999; Ji et al., 2014; Mann et al., 2016b). As a consequence, overfeeding

during a timespan similar to the duration of the dry period, increases adipocyte size, BCS and abdominal fat mass (Drackley et al., 2014; Locher et al., 2015). After calving, when energy balance is comparable between cows, body fatness or adipocyte size will be the main regulator of the lipolytic activity of adipose tissue. We hypothesize that overfed and overconditioned dairy cows have larger adipocytes and in lipolytic conditions, the metabolic properties of these large adipocytes are characterized by an increased basal and catecholamine stimulated lipolytic activity whereas the inhibitory effect of insulin on the lipolytic activity is preserved.

2.4. The pro-inflammatory state in overconditioned cows

All dairy cows have elevated serum concentrations of inflammatory mediators in the first days post partum due to the fact that parturition and expulsion of the placenta are inflammatory processes (Bertoni et al., 2008; Qu et al., 2014; Bradford et al., 2015). While a normal inflammatory reaction is necessary and beneficial in this period to overcome adverse inflammatory stimuli (tissue damage, bacteria, ...), dysregulated inflammatory processes will not resolve the inflammatory trigger and are harmful for the metabolism of cells and tissues (Bradford et al., 2015). A normal inflammatory reaction involves many cell types and tissues acting both locally and systemically, and will resolve the inflammatory stimulus and tissue damage (Sordillo and Raphael, 2013; Bradford et al., 2015). Dysregulated inflammatory processes are uncontrolled inflammatory reactions towards an inflammatory trigger which can be either too little (hypo-responsive inflammation), too much (hyper-responsive inflammation) or too long in duration (subacute or chronic inflammation) (LeBlanc, 2012; Sordillo and Raphael, 2013; Bradford et al., 2015).

There are different underlying reasons for the dysregulation of the inflammatory processes in the periparturient dairy cow which have been reviewed by Sordillo and Raphael (2013) and Bradford et al. (2015). Besides the direct role of NEFA on transition cow disorders, excessive NEFA and BHB have a negative impact on immune cell function and elevated concentrations of saturated fatty acids, as observed in the periparturient period, may activate the inflammatory signalling cascade (Contreras and Sordillo, 2011). Oxidative stress, due to an imbalance between the production of reactive oxygen species and antioxidants, reduces functional activity of leukocytes and activates the inflammatory signalling cascade through NF κ B (Sordillo and

Aitken, 2009; Sordillo and Raphael, 2013). The periparturient period is characterized by higher levels of ROS due to the increased peroxisomal β -oxidation of NEFA in the liver (Sordillo and Raphael, 2013; Bradford et al., 2015). Sudden ration changes, like the shift from a high-forage dry cow ration to a high-grain lactating cow ration, are associated with a decrease of ruminal pH and changes of the ruminal flora. In those conditions, gram negative bacteria die or proliferate and LPS is released in the rumen (Plaizier et al., 2008; Eckel and Ametaj, 2016). It is supposed that this LPS activates the inflammatory cascade in the transition dairy cow (Eckel and Ametaj, 2016). Additional factors that have been suggested to play a role in inflammatory processes in the periparturient dairy cow are endoplasmic reticulum stress, social stress, heat stress and subclinical diseases (mastitis and metritis) (Proudfoot et al., 2012; Bradford et al., 2015; Ringseis et al., 2015; Eckel and Ametaj, 2016). A final factor which is considered to have a causative role in the dysregulation of inflammatory processes in the periparturient period is the adipose tissue (Vernon, 2002; Drackley et al., 2005; Loores et al., 2007).

In Chapter 7.1, we investigated the potential role of the adipose tissue in the dysregulation of inflammatory processes in overconditioned cows. By measuring the mRNA expression of different adipokines and CD14 in different adipose depots from cows with a variable BCS at the end of pregnancy, we determined the influence of adipocyte size and adipose depot on the expression of adiponectin, leptin, IL6, TNF and CD14. Depots with large adipocytes had higher expression of CD14 which is a marker for macrophages. This suggests that the adipose tissue of overconditioned cows is infiltrated with macrophages. The positive association between the expression of CD14 and IL6 and TNF suggests furthermore that the macrophages were pro-inflammatory polarized. Especially adipose depots with large adipocytes demonstrated higher expression of CD14, IL6 and TNF. In addition, leptin is able to activate the pro-inflammatory cascade and was positively associated with adipocyte size. The results suggest a pro-inflammatory state of the adipose depots in overconditioned cows. However, it should be noted that we did not measure the expression of anti-inflammatory cytokines. The expression of anti-inflammatory cytokines might be upregulated in adipose depots with large adipocytes as a compensation mechanism. Therefore more research is needed to investigate the expression of additional cytokines like IL10.

On the other hand, adiponectin expression was not influenced by differences in adipocyte size. It has been described that adiponectin production by the adipose tissue is mainly controlled by post-transcriptional factors (Lemor et al., 2009; Singh et al., 2014). The serum adiponectin concentrations of the cows in the present study were negatively associated with the BCS of the animals (Chapter 7.2), confirming the post-transcriptional regulation of adiponectin production. We acknowledge that the results of Chapter 7.1 reflect changes of expression of genes measured at the mRNA level and thus prone to post-transcriptional regulation influencing protein abundance and activity. Therefore, more research is warranted to confirm our results at the protein level of the adipose tissue using western blot for leptin, adiponectin, IL6 and TNF and using immunohistochemistry to detect adipose tissue macrophages (ATM). Previous research on ATM did not demonstrate an appreciable infiltration of ATM in early lactating dairy cows, overconditioned heifers or fat bulls (Akter et al., 2012). Contrary, in cows with LDA, which are metabolically triggered, the subcutaneous and omental adipose tissue demonstrated a marked infiltration of ATM (Contreras et al., 2015). Although the expression of CD14 is not a direct measure of ATM, the results of Chapter 7.1 confirm the observation of Contreras et al. (2015).

In general, internal adipose depots demonstrated higher expression of pro-inflammatory adipokines compared with the subcutaneous adipose depot. Similarly, the omental adipose tissue expressed more pro-inflammatory markers compared with the subcutaneous adipose tissue of LDA cows (Contreras et al., 2015). In the study of Saremi et al. (2014), in early lactating dairy cows, most differences were observed in the retroperitoneal adipose tissue. The difference between studies may be due to the use of different methodologies or due to differences in experimental animals. Compared with the lactating cows, the overconditioned and the LDA cows are exposed to a higher metabolic load which might cause a cascade of detrimental consequences.

Based on our observations and the similarities with the human metabolic syndrome, we hypothesize that the production of pro-inflammatory adipokines in the internal adipose depots, will trigger the liver to produce pro-inflammatory mediators and acute phase proteins. This will induce a chronic inflammatory state that dysregulate inflammatory processes and increase the

risk for infectious and metabolic disorders (Figure 5). This hypothesis could not be confirmed in the study of Akbar et al. (2015) in which cows at the end of lactation with high (5.0 on a 10 point scale), medium (4.0 on a 10 point scale) and low BCS (3.0 on a 10 point scale) were sampled throughout the periparturient period. However, high BCS cows in the latter study (5.0 on a 10 point scale) were not as fat as the cows in our study (5.0 on a 5 point scale). As such the cows in the study of Akbar et al. (2015) might not have been triggered to experience the negative consequences of an excessive accumulation of body fat.

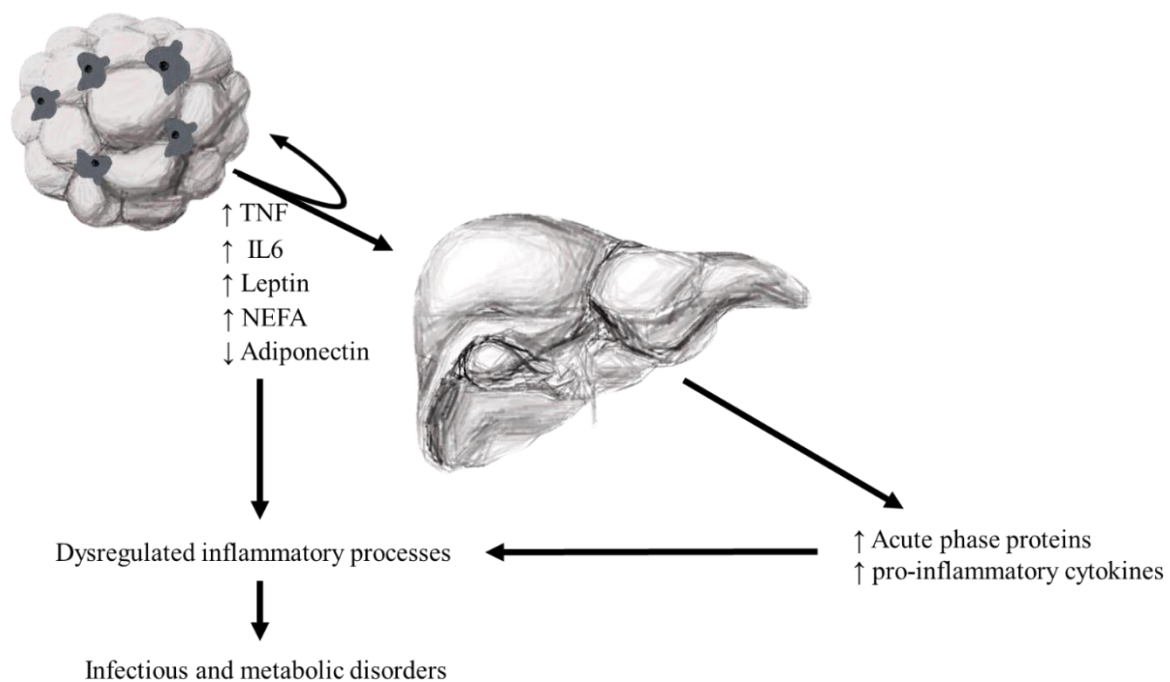


Figure 5: Hypothesized relationship between the production of pro-inflammatory adipokines by the adipose tissue and dysregulation of inflammatory processes.

A link between pro-inflammatory cytokines (TNF) and fatty liver is clearly established (Ohtsuka et al., 2001; Bradford et al., 2009). Subcutaneous daily injections of TNF will lead to an obvious infiltration of TG in hepatocytes. When an identical dose of TNF is given as a continuous low dose infusion into subcutaneous adipose tissue, similar to what would happen when the adipose tissue is in a pro-inflammatory state, no hepatic TG infiltration occurs and anti-inflammatory reactions are upregulated in liver and adipose tissue (Martel et al., 2014). Based on these observations, the role of the adipose tissue in the dysregulation of inflammatory processes is questioned. It is not known if the chronic locally increased production of pro-inflammatory adipokines may reach the systemic circulation and affect metabolic and

inflammatory processes. On the other hand it is questioned if overconditioned cows can be considered ‘obese’: in human obesity, the relative amount of body fat is much higher (40%) compared with the body fat content of dairy cows (20%) (Bradford et al., 2015). In addition, it can be questioned if the relatively short-term effect of overconditioning in dairy cows, occurring at the end of lactation and during the dry period, is long enough in duration to have a detrimental impact on metabolic and inflammatory processes. Typically, overconditioning is automatically corrected at the beginning of lactation when cows start to mobilize adipose tissue. In contrast, humans are obese for years before experiencing negative health effects. Therefore, more research is needed to determine the potential relationship between the expression of pro-inflammatory molecules in adipose tissue of overconditioned cows, the inflammatory profile in these cows and possible health consequences.

3. CONCLUSIVE REMARKS AND FUTURE RESEARCH PERSPECTIVES

In the present thesis, we investigated the use of different methods to measure insulin resistance in dairy cows. We came to the conclusion that surrogate indices for insulin sensitivity are not useful in dry dairy cows and require further validation before being used in lactating dairy cows. When comparing cows in the same physiological state, the HEC test, the GTT, the ITT, and in vitro and in vivo sampling of tissues after activation of the insulin signaling cascade are useful methods. When comparing cows in a different physiological state, the assessment of insulin resistance is hampered by the insulin independent glucose uptake of the mammary gland and/or the pregnant uterus. The insulin independent glucose uptake is very difficult to quantify unless one utilizes isotopes of glucose during HEC tests (Rose et al., 1997; Weber et al., 2016). An alternative for the use of the isotopes, is by measuring tissue-specific insulin signaling by in vitro or in vivo sampling of tissues after activation of the insulin signaling cascade (Ji et al., 2012; Zachut et al., 2013; Mann et al., 2016a; Mann et al., 2016b). The latter method is preferred to be able to distinguish between the activation of different insulin sensitive metabolic pathways. Because, as demonstrated in Chapter 5, the insulin sensitivity of one metabolic pathway (e.g. glucose metabolism) may differ from another metabolic pathway (e.g. NEFA metabolism). Further optimization of studies investigating tissue-specific insulin signaling can be achieved by using standardized insulin boluses.

Overconditioned cows are prone to metabolic and infectious disorders in the periparturient period. This is not due to one specific factor but rather the cumulative effect of different stressors (NEFA, adipokines) which act acutely or chronically. Once the critical threshold is reached, the cows go in a downward spiral and will suffer from different metabolic and infectious disorders (Drackley et al., 2005). In the present study we tried to identify factors that contribute to the increased disease susceptibility of overconditioned cows. Based on our results, especially the high accumulation of fat, the lipolytic activity of enlarged adipocytes (in vitro) and the dysregulated production of adipokines by the adipose tissue, seem to contribute to the fat cow syndrome. Although our results contributed to the understanding of the fat cow syndrome, there were some limitations in our experiments, providing opportunities for further research:

- Overconditioning during the dry period is associated with an insulin resistant state of the glucose metabolism but not of the NEFA metabolism. Thus, the NEFA lowering effect of insulin is maintained in overconditioned dairy cows at the end of the dry period. Our results were obtained in dairy cows at the end of pregnancy, it would be interesting to verify if a similar association between fat accumulation and insulin resistance is also existing in the immediate postpartum period.
- In vitro research of adipose tissue explants confirmed the fact that the inhibitory effect of insulin on lipolysis is not affected by excessive fat accumulation. However, enlarged adipocytes from overconditioned cows have higher basal and stimulated lipolytic activity. Similar to the study of the HEC test, these results were obtained in dairy cows at the end of pregnancy, as such, more research is needed to verify if a similar association between adipocyte size and basal, stimulated and inhibited lipolytic activity is also existing in the immediate postpartum period.
- We provided evidence that the lipolytic activity of adipocytes depends on the localization of the adipose depots. It would be of particular interest to involve more depots than merely the subcutaneous and omental depots and to investigate if the lipolytic activity changes dependent on the adipocyte size and adipose depot localization during lactation.

- The preservation of the NEFA lowering effect of insulin is favorable because if cows maintain sufficient insulin and/or glucose levels in the periparturient period, insulin is effective in reducing circulating NEFA (van Knegsel et al., 2007; Lucy et al., 2013). However, basal and stimulated insulin concentrations are highly variable between cows. Of particular interest is the fact that ketotic and feed deprived cows (Hove, 1978; Schoenberg et al., 2012) have lower circulating insulin concentrations. However, other factors, like DMI, prepartum ration and the starch and NDF content of the postpartum ration are expected to influence circulating insulin concentrations as well (Dann et al., 2006; Bradford and Allen, 2007; Boerman et al., 2015; McCarthy et al., 2015b). Future investigations should focus on physiological and pathological factors that may explain variation in basal and stimulated insulin secretion in the periparturient period with special attention to the influence of pre- and postpartum ration on basal insulin levels.
- In Chapter 7.1, mRNA expression was determined which is prone to posttranscriptional modifications. More research is warranted to confirm our results at the protein level of the adipose tissue using western blot for leptin, adiponectin, IL6 and TNF and using immunohistochemistry to detect ATM.
- It is questioned if the locally increased production of pro-inflammatory cytokines in adipose depots has a negative influence on metabolic and inflammatory pathways. More research is warranted to identify the potential relationship between the expression of pro-inflammatory molecules in adipose tissue of overconditioned cows, the inflammatory profile of these cows and potential health consequences.

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Summary

Maintaining glucose homeostasis is the principal challenge a dairy cow faces in the periparturient period. In **Chapter 1**, we described how the metabolism of the cow adapts to preserve sufficient glucose for the growing fetus or nursing neonate. Cows ruminate and absorb little glucose directly from the gastrointestinal tract, as such, the glucose metabolism between ruminants and most other mammals is very different. Glucose in ruminants is mainly supplied by hepatic gluconeogenesis starting from volatile fatty acids (with propionate being the most important), lactate, amino acids and glycerol. At the end of pregnancy, the glucose requirement of a dairy cow was estimated to be 1,000 g per day. Sixty to seventy percent of this glucose is shifted to the pregnant uterus. After delivery, the glucose requirement of an average producing cow increases up to 3,500 g per day. More than eighty percent of this glucose is preserved for the lactating mammary gland. This large shift in glucose requirements during the transition period is regulated by changes in circulating hormone concentrations and changes in tissue sensitivity to these hormones. The end result is increased hepatic glucose production, decreased glucose use by peripheral tissues and increased use of energy derived from lipids. The metabolic adaptations occurring during the transition period makes dairy cows more vulnerable to metabolic and infectious diseases. Almost half of the dairy cows suffer from one or more transition disorders. Overconditioning is an important risk factor for the derailment of the normal metabolism in the transition period and all too often leads to a disease known as ‘the fat cow syndrome’. In **Chapter 2**, we reviewed the current knowledge about the fat cow syndrome and compared it with the human metabolic syndrome. The human metabolic syndrome is a disorder in which obesity is associated with insulin resistance, type 2 diabetes mellitus and cardiovascular disease. In this chapter, special attention was paid to the role of insulin resistance in the metabolic adaptation of dairy cows to preserve glucose for the pregnant uterus and lactating mammary gland.

The aim of the present thesis was to assess the role of the adipose tissue in the disease susceptibility and in the development of insulin resistance in overconditioned dairy cows (**Chapter 3**). The assessment of insulin resistance in pregnant and lactating dairy cows is complicated by the fact that large amounts of glucose disappear independently of insulin. In the first part of our research (**Chapter 4**), we validated and evaluated different methods to

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assess insulin resistance of the glucose metabolism in dairy cows in a lactating or pregnant state. In **Chapter 4.1**, the glucose tolerance test and surrogate indices for insulin sensitivity were validated with the hyperinsulinemic euglycemic clamp (HEC) test in dairy cows at the end of pregnancy. The HEC test is the gold standard to assess insulin resistance in humans and animals, yet is very laborious and time consuming. The surrogate indices for insulin sensitivity are adopted from human medicine and are based on mathematical formulas including glucose, insulin, NEFA and/or BHB concentrations measured in a single blood sample. In Chapter 4.1, we demonstrated that the surrogate indices for insulin sensitivity were not associated with measures of insulin sensitivity derived from HEC tests. However, two measures of insulin sensitivity derived from the glucose tolerance test (GTT) demonstrated a good correlation with the HEC test: the area under the curve for glucose for the first 60 min after the glucose infusion and the insulin sensitivity index, S_i , derived from the minimal model. Hence, these can be used as reliable measures of insulin sensitivity in dairy cows at the end of pregnancy. In **Chapter 4.2**, we aimed to determine the impact of insulin independent processes, especially glucose uptake by the pregnant uterus or lactating mammary gland, on insulin sensitivity parameters derived from GTT. By comparing nonlactating-nonpregnant heifers with pregnant or lactating heifers, we were able to see the impact of different physiological states on GTT. Glucose clearance and S_i were lower while the AUC of glucose was higher in the nonpregnant-nonlactating heifers compared with both the pregnant and the lactating heifers. The latter is indicative for reduced glucose tolerance in the nonpregnant-nonlactating heifers. The reduced glucose tolerance can be attributed to a decreased peripheral tissue insulin sensitivity of the glucose metabolism (insulin dependent glucose disappearance) or a decreased insulin independent glucose disappearance in nonpregnant-nonlactating heifers. Based on the results from a GTT, it is impossible to discriminate between insulin independent and insulin dependent glucose disappearance. Therefore, GTT are not indicated to compare insulin resistance between heifers or cows in different physiological states.

In **Chapter 5**, the HEC test was used to identify the impact of excessive fat accumulation on insulin sensitivity in dairy cows at the end of the dry period. Ten cows were selected based on body condition score and HEC tests were performed at the end of the dry period. By measuring

both glucose and NEFA during the HEC test, we were able to determine the insulin sensitivity of the glucose and fatty acid metabolism of cows differing in BCS. Insulin sensitivity and insulin responsiveness of the glucose metabolism were negatively associated with BCS, backfat thickness and fat accumulation in different fat depots of the body. Interestingly, insulin sensitivity and insulin responsiveness of the NEFA metabolism were not associated with the level of body fatness. It was concluded that fat accumulation in dairy cows at the end of the dry period, negatively influences insulin sensitivity and insulin responsiveness of the glucose metabolism while insulin action at the level of the NEFA metabolism is preserved. Additionally, we demonstrated that the insulin concentration needed to achieve the halfmaximal effect was different between the glucose and NEFA metabolism. The effect of insulin on the NEFA metabolism was reached at lower insulin concentrations compared with the glucose metabolism. This means that when insulin concentrations decrease, like at the end of pregnancy and the beginning of lactation, uptake of glucose in the insulin sensitive peripheral tissues will decrease first, before dairy cows start to mobilize NEFA.

In **Chapter 6**, we specifically investigated the in vitro lipolytic activity of adipocytes from two different adipose depots, the subcutaneous and the omental adipose depot. The basal lipolytic activity of adipose tissue fragments was higher in larger adipocytes but not different between the two depots. After stimulation of the lipolytic activity with a β agonist, lipolytic activity increased more in larger adipocytes and more in the subcutaneous compared with the omental adipose tissue. Due to the higher basal and stimulated lipolytic activity of large adipocytes, overconditioned cows are prone to excessive mobilization of body fat and will have elevated levels of NEFA. When the lipolytic activity of the adipocytes was inhibited with insulin, large and small adipocytes reacted in a similar way and there was no difference between the two depots. This means that, similar to the in vivo observations in Chapter 5, the inhibitory effect of insulin on NEFA release is preserved in overconditioned cows.

Besides the release of NEFA, the adipose tissue has the capacity to secrete different adipokines. The aim of **Chapter 7** was to determine the association between the expression of different adipokines and the level of fat accumulation in dairy cows at the end of pregnancy. Using RT-qPCR, mRNA expression of adiponectin, leptin, IL6 and CD14 was determined in the

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subcutaneous, mesenteric, omental, perirenal and intrapelvic adipose depots (**Chapter 7.1**). Adipocyte size was positively correlated with the BCS in all depots, which indicates that accumulation of lipids occurred mainly via hypertrophy of the adipocytes. The expression of adiponectin was not influenced by adipocyte size, however, the serum concentrations of adiponectin were positively associated with the BCS of the animals (**Chapter 7.2**). The difference between the expression and the circulating levels of adiponectin reflects that the production of adiponectin is mainly mediated via post-transcriptional factors. Furthermore, expression of pro-inflammatory adipokines (leptin and IL6) and CD14 was positively associated with adipocyte size. This is indicative for the fact that the adipose depots of overconditioned cows are infiltrated by macrophages and that these macrophages are pro-inflammatory activated. Generally, the internal adipose depots (mesenteric, omental, perirenal and intrapelvic) demonstrated a more pro-inflammatory expression profile compared with the subcutaneous adipose depots. All together, the expression profile is indicative for a pro-inflammatory state of the adipose depots from overconditioned cows which might dysregulate inflammatory processes and contribute to metabolic and infectious disorders in the periparturient period.

In **Chapter 8**, we indicated opportunities for further research and the results of the previous chapters were discussed in a broader scientific perspective. The most important conclusions were:

- Assessment of insulin sensitivity of the glucose metabolism is very challenging, especially between cows in a different physiological state. The large difference between insulin independent glucose disappearance via the gravid uterus and lactating mammary gland, makes methods measuring glucose disappearance (GTT and ITT) useless. More useful methods are the HEC test in combination with stable isotopes of glucose or measuring tissue-specific insulin signaling in tissue biopsies taken before and after an insulin challenge.
- Overconditioned dairy cows at the end of pregnancy are insulin resistant at the level of the glucose metabolism but not at the level of the NEFA metabolism. This suggests that the NEFA lowering effect of insulin is preserved in overconditioned cows.

- The insulin concentration needed to achieve the halfmaximal effect of insulin is lower for the NEFA metabolism compared with the glucose metabolism. This implicates that the effect of a certain concentration of insulin will be greater at the level of the NEFA metabolism compared with the glucose metabolism.
- Overconditioned dairy cows at the end of pregnancy are destined to be exposed to high levels of NEFA in the periparturient period due to different factors. Overconditioned cows have more body fat and this will release more NEFA upon stimulation. Adipocytes from overconditioned cows are larger and the metabolic properties of these large adipocytes are characterized by an increased basal and catecholamine stimulated lipolytic activity. Comparable to the results in Chapter 5, the inhibitory effect of insulin on the lipolytic activity of adipocytes is preserved in overconditioned cows.
- Overconditioned dairy cows are more susceptible to dysregulation of inflammatory processes. The adipose tissue of overconditioned cows might play a central role by the production of pro-inflammatory adipokines (leptin and IL6) and the infiltration of macrophages in the adipose tissue.

Samenvatting

Het in stand houden van de glucose homeostase is de belangrijkste uitdaging voor een melkkoe in de transitieperiode. In **Hoofdstuk 1** beschrijven we hoe het metabolisme van de melkkoe zich aanpast om de groeiende foetus en vervolgens het zogende kalf van voldoende glucose te voorzien. Het glucose metabolisme van herkauwers en éénmagigen verschilt sterk van elkaar omdat koeien herkauwers zijn en daardoor weinig glucose rechtstreeks opnemen vanuit hun maagdarmsstelsel. Herkauwers worden voorzien van glucose via de gluconeogenese in de lever. De metabolieten die de lever hiervoor gebruikt zijn: vluchtige vetzuren (voornamelijk propionzuur), melkzuur, aminozuren en glycerol. De glucose behoefte van een melkkoe op het einde van de dracht wordt geschat op 1,000 gram per dag. Ongeveer zestig tot zeventig percent van deze glucose wordt opgenomen door de drachtige baarmoeder. Na de kalving stijgt de glucose behoefte van een lacterende koe tot ongeveer 3,500 gram per dag. Meer dan tachtig procent van deze glucose is voorbehouden voor de uier. De specifieke veranderingen in het glucose metabolisme tijdens de transitieperiode worden gestuurd door veranderingen in de concentraties van hormonen en veranderingen in de reacties van de weefsels op deze hormonen. Het eindresultaat hiervan is een verhoogde glucose productie door de lever en een verminderd glucose verbruik door de perifere weefsels die eerder vrije vetzuren (NEFA) of ketonlichamen (BHB) als energiebron zullen gebruiken. De metabole veranderingen in de transitieperiode, zorgen er echter voor dat melkkoeien gevoeliger zijn voor metabole en infectieuze aandoeningen in deze periode. Bijna de helft van alle melkkoeien wordt geconfronteerd met één of meerdere transitieproblemen. Vervetting is een belangrijke risicofactor voor het ontsporen van het normale metabolisme in de transitieperiode en geeft vaak aanleiding tot het ‘fat cow syndroom’. In **Hoofdstuk 2** hebben we de huidige kennis over het ‘fat cow syndroom’ beschreven en vergeleken met het humaan metabool syndroom. Het humaan metabool syndroom is een aandoening waarin obesitas wordt geassocieerd met insulineresistentie, type 2 diabetes mellitus en cardiovasculaire aandoeningen. In dit hoofdstuk zijn we ook dieper ingegaan op de rol die insulineresistentie speelt in het metabolisme van koeien in de transitieperiode.

Het doel van dit doctoraatswerk was om te bepalen welke rol het vetweefsel speelt in de ziektegevoeligheid en de ontwikkeling van insulineresistentie bij melkkoeien die te vet zijn op

het einde van de dracht (**Hoofdstuk 3**). Het bepalen van insulineresistentie bij drachtige en lacterende koeien is een uitdaging omwille van het feit dat grote hoeveelheden glucose onafhankelijk van insuline verdwijnen via de drachtige baarmoeder of de uier. In het eerste deel van dit onderzoek (**Hoofdstuk 4**) hebben we verschillende methoden om insulineresistentie van het glucose metabolisme te meten bij melkkoeien uitgetest, met elkaar vergeleken en geëvalueerd. In **Hoofdstuk 4.1**, werden de glucose tolerantie test (GTT) en verschillende surrogaat indexen om de insulinegevoeligheid in te schatten, vergeleken met de hyperinsulinemische euglycemische clamp (HEC) test bij melkkoeien op het einde van de dracht. De hyperinsulinemische euglycemische clamp test is de gouden standaard om insulineresistentie in te schatten bij mens en dier, maar is heel arbeidsintensief en tijdrovend. De surrogaat indexen om insulinegevoeligheid in te schatten, zijn overgenomen uit de humane geneeskunde en zijn mathematische berekeningen van een index gebaseerd op de glucose, insuline, NEFA en/of BHB concentratie gemeten in één enkel bloedstaal. Deze surrogaat indexen om insulinegevoeligheid in te schatten, bleken niet geassocieerd te zijn met de insulinegevoeligheid bepaald door de HEC test. Echter, de oppervlakte onder de curve van glucose voor de eerste 60 min na een glucose infuus en de insulinegevoelighedsindex, S_i , afgeleid van het minimal model, bleken een goede correlatie te hebben met de resultaten van de HEC test. Deze kunnen dus gebruikt worden om de insulinegevoeligheid in te schatten bij melkkoeien op het einde van de dracht. Het doel van **Hoofdstuk 4.2** was om te bepalen wat de invloed is van insuline onafhankelijke processen, voornamelijk glucose opname door de drachtige baarmoeder of uier, op insulinegevoelighedsparameters afgeleid van de glucose tolerantie test. Door niet-drachtige, niet-lacterende vaarzen te vergelijken met drachtige of lacterende vaarzen, waren we in staat om de impact te bepalen van dracht en lactatie op de resultaten van een GTT. De klaring van glucose en S_i waren lager, terwijl de oppervlakte onder de curve van glucose hoger was bij de niet-drachtige, niet-lacterende vaarzen in vergelijking met de drachtige vaarzen en de lacterende vaarzen. Dit duidt op een verminderde glucose tolerantie bij de niet-drachtige, niet-lacterende vaarzen. De verminderde glucose tolerantie kan te wijten zijn aan verminderde perifere insulinegevoeligheid van het glucose metabolisme (insuline afhankelijk glucose metabolisme) of verminderde insuline onafhankelijke

verdwijning van glucose in de niet-drachtige, niet-lacterende vaarzen. Het is onmogelijk om op basis van de resultaten van de GTT een onderscheid te maken tussen insuline afhankelijke en insuline onafhankelijke processen. Daarom is de GTT niet aangewezen om de mate van insulineresistentie te vergelijken tussen koeien en vaarzen in een verschillende fysiologische toestand (dracht versus lactatie).

In **Hoofdstuk 5** werd de HEC test uitgevoerd bij melkkoeien op het einde van de dracht om in te schatten wat de invloed was van overmatige vervetting op de insulinegevoeligheid. Tien koeien werden geselecteerd op basis van hun sterk uiteenlopende conditiescore en de HEC test werd uitgevoerd op het einde van de droogstand. Door zowel glucose als NEFA te bepalen tijdens de HEC test, waren we in staat om de insulinegevoeligheid van zowel het glucose als het NEFA metabolisme te bepalen bij melkkoeien met verschillende conditiescores. Insulinegevoeligheid en insulinerespons van het glucose metabolisme waren negatief geassocieerd met de conditiescore, rugvetdikte en vetopstapeling in verschillende vetdepots. De insulinegevoeligheid en de insuline respons van het NEFA metabolisme waren niet geassocieerd met de vetheid van de dieren. Als conclusie werd gesteld dat vetopstapeling bij melkkoeien op het einde van de droogstand, een negatieve invloed heeft op zowel de insulinegevoeligheid als de insuline respons van het glucose metabolisme terwijl het effect van insuline op het NEFA metabolisme behouden blijft. Daarenboven toonden we aan dat de insuline concentratie, nodig om het halfmaximaal effect te bereiken, verschillend was tussen het glucose en NEFA metabolisme. Het effect van insuline op het NEFA metabolisme werd bereikt bij een lagere insuline concentratie in vergelijking met het glucose metabolisme. Dit betekent dat wanneer de insulineconcentratie daalt, zoals op het einde van de dracht en aan het begin van de lactatie, de opname van glucose in de perifere weefsels eerst zal verminderen vooraleer de koeien zullen beginnen met het mobiliseren van NEFA.

In **Hoofdstuk 6** onderzochten we de in vitro lipolytische activiteit van vetcellen van twee verschillende vetdepots, het subcutaan en omentaal vetdepot. De basale lipolytische activiteit was groter in grote vetcellen maar niet verschillend tussen de twee depots. Na stimulatie van de lipolytische activiteit met een β agonist, nam de lipolytische activiteit meer toe in grotere vetcellen en in het subcutaan vetweefsel. Door de grotere basale en gestimuleerde lipolytische

activiteit van grotere vetcellen, zullen vette koeien meer gevoelig zijn voor overmatige mobilisatie van vetweefsel en daardoor verhoogde concentraties van NEFA in het bloed hebben. Grote en kleine vetcellen reageerden op dezelfde manier wanneer de lipolytische activiteit van de vetcellen werd geïnhibeerd met insuline. Dit betekent dat, vergelijkbaar met de in vivo resultaten van Hoofdstuk 5, de inhibitorische activiteit van insuline op de vrijstelling van NEFA uit het vetweefsel, behouden blijft bij vette koeien.

Naast de vrijstelling van NEFA, is het vetweefsel ook in staat om verschillende adipokines te produceren. In **Hoofdstuk 7** was het de bedoeling om de associatie te bepalen tussen de expressie van verschillende adipokines en de mate van vetopstapeling bij melkkoeien op het einde van de dracht. Door middel van RT-qPCR, werd de mRNA expressie van adiponectine, leptine, IL6 en CD14 bepaald in subcutaan, omentaal, mesenteriaal, perirenaal en intrapelvien vet (**Hoofdstuk 7.1**). In alle vetdepots was de grootte van de vetcellen positief gecorreleerd met de conditiescore van de koeien, hetgeen erop wijst dat de vetopstapeling voornamelijk gebeurde via hypertrofie van de vetcellen. De expressie van adiponectine werd niet beïnvloed door de grootte van de vetcellen. Echter, de serumconcentratie van adiponectine was positief gecorreleerd met de conditiescore van de dieren (**Hoofdstuk 7.2**). Het verschil tussen de expressie en de circulerende concentratie van adiponectine, duidt erop dat de productie van adiponectine voornamelijk geregeld wordt via post-transcriptionele factoren. De expressie van de pro-inflammatoire adipokines (leptine en IL6) en CD14 was positief geassocieerd met de grootte van de vetcellen. Dit duidt erop dat de vetdepots van vette koeien geïnfiltreerd waren met macrofagen en dat deze macrofagen een pro-inflammatoir expressieprofiel vertoonden. Over het algemeen hadden de inwendige vetdepots (mesenteriaal, omentaal, perirenaal en intrapelvien) een meer pro-inflammatoir expressieprofiel in vergelijking met het subcutaan vetdepot. Als conclusie werd gesteld dat de vetdepots van vette koeien gekenmerkt zijn door een pro-inflammatoir expressieprofiel wat kan leiden tot ontregeling van inflammatoire processen en aldus kan bijdragen tot de ontwikkeling van metabole en infectieuze aandoeningen in de transitieperiode.

In **Hoofdstuk 8** werden de resultaten uit de voorgaande hoofdstukken in een breder wetenschappelijk kader bediscussieerd en werden mogelijkheden voor verder onderzoek aangegeven. De belangrijkste conclusies waren:

- Het inschatten van de insulinegevoeligheid van het glucose metabolisme is heel uitdagend bij melkkoeien, zeker wanneer men drachtige en lacterende dieren wil vergelijken. Het grote verschil in insuline onafhankelijke glucose opname tussen de drachtige baarmoeder en de uier, zorgt ervoor dat procedures die gebaseerd zijn op het meten van de verdwijning van glucose (GTT en ITT), niet kunnen gebruikt worden. Andere procedures, zoals de HEC test in combinatie met het gebruik van stabiele isotopen van glucose of het meten van de activatie van de insuline signaal cascade in weefselstaaltjes genomen voor en na het stimuleren van de insuline signaal cascade met insuline verdienen hiertoe de voorkeur.
- Vette koeien zijn op het einde van de dracht insulineresistent. Deze insulineresistentie situeert zich op het niveau van het glucose metabolisme maar niet ter hoogte van het NEFA metabolisme. Dit suggereert dat het effect van insuline om de NEFA concentratie te doen dalen, behouden blijft bij vette koeien.
- De insulineconcentratie nodig om het halfmaximaal effect te bereiken, is lager voor het NEFA metabolisme in vergelijking met het glucose metabolisme. Hierdoor zal het effect van een bepaalde hoeveelheid insuline groter zijn op het niveau van het NEFA metabolisme dan op dat van het glucose metabolisme.
- Vette koeien zijn voorbestemd om hoge gehalten aan NEFA te hebben in de transitieperiode omwille van verschillende factoren. Vette koeien hebben meer lichaamsvet en zullen daardoor meer NEFA mobiliseren wanneer het vetweefsel gestimuleerd wordt. Vetcellen van vette koeien zijn bovendien groter en de metabole eigenschappen van deze grote vetcellen zijn gekenmerkt door een grotere basale en gestimuleerde lipolytische activiteit. Het inhiberend effect van insuline op de lipolyse blijft behouden bij vette koeien.

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- Vette koeien zijn gevoeliger voor de ontregeling van inflammatoire processen. Het vetweefsel speelt hierin een rol door de productie van pro-inflammatoire adipokines (leptine en IL6) en door de infiltratie van macrofagen in het vetweefsel.

Curriculum vitae

Jenne De Koster werd geboren op 11 maart 1985 te Anderlecht. Na het behalen van het diploma hoger secundair onderwijs in het Sint-Jozefsinstituut te Ternat in de richting Wetenschappen-Wiskunde, startte hij in 2003 met de studie Diergeneeskunde aan de Universiteit Gent. In 2009 behaalde hij het diploma van Dierenarts (optie Herkauwers) met grote onderscheiding.

Na het afstuderen, werkte hij een jaar in de dierentuin Park Paradisio om vervolgens in dienst te treden aan de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. Gedurende 4 jaar werd hij gefinancierd door het Bijzonder Onderzoeksfonds van de Universiteit Gent om als doctoraatsstudent onderzoek te verrichten naar de factoren die een rol spelen in de ontwikkeling van insuline resistentie en verhoogde ziektegevoeligheid bij melkvee in de transitieperiode, onder begeleiding van Prof. Dr. Geert Opsomer, Prof. Dr. Wim Van den Broeck en Dr. Miel Hostens. In 2015 werd hij voltijds assistent bij de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. Tot eind 2016 bestond zijn taak erin om dit doctoraatswerk af te werken, onderwijs te geven aan de laatstejaarsstudenten en dienstverlening te voorzien bij klanten van de Buitenpraktijk en Bedrijfsbegeleiding. Hij was actief betrokken bij de dag-, nacht- en weekenddiensten in de Buitenpraktijk en de kliniek Verloskunde van de vakgroep.

Jenne De Koster is auteur en medeauteur van diverse wetenschappelijke publicaties in verschillende nationale en internationale tijdschriften en gaf presentaties op verschillende nationale en internationale congressen en symposia.

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Dankwoord

De opeenvolging van woorden in dit doctoraat heeft min of meer een zekere betekenis maar wat nog meer betekenis heeft, zijn de mensen die mijn pad hebben gekruist de voorbije 6 jaren. Veel positieve ervaringen meegemaakt, ook negatieve maar die zijn we alweer vergeten, selectief geheugen ☺ of te kleine hersenen om alles te onthouden.

Beginnen doen we bij de belangrijkste mens die er vanaf de eerste tot de laatste dag bij betrokken is geweest, professor Opsomer. Geert, ik vermoed dat ook u af en toe met de handen in het haar hebt gezeten tijdens de voorbije 6 jaar: omwille van proefopzetten die finaal afgekeurd werden door het FAVV, koeien die te vroeg kaldden, de hoge prijs van de reforme koeien op het moment dat ik proefkoeien nodig had of als ik weer eens kwam klagen dat mijn proeven in het labo mislukten om de één of andere onverklaarbare reden. Ik heb veel te danken aan de vrijheid die u mij hebt gegeven tijdens dit doctoraat. Grotendeels moest ik zelf op zoek gaan naar de juiste manier van proeven uitvoeren met veel ups en downs. U zei echter altijd dat dit op het einde van de rit als positief wordt beschouwd door de doctorandus en op dit moment kan ik dat alleen maar beamen. We hebben veel goeie momenten meegemaakt, niet alleen het congres in Turkije (waar u toch tot in de vroege uurtjes hebt meegefeest, of was dit alleen maar om ons in de gaten te houden zodat we niks verkeerd zouden doen?), de bbq's bij u thuis of op Axelwalle als teambuilding, het reisje naar Macedonië voor Branko zijn doctoraat, ons bezoekje aan Cambridge... Ik heb me geamuseerd en ben blij dat u me begeleid heeft de voorbije jaren. Bedankt!

Professor Van den Broeck, Wim, mijn bezoeken aan de vakgroep Morfologie waren vaak nogal onverwacht maar dankzij uw steun en de goede samenwerking met de collega's op de vakgroep, Prof. Simoens, Prof. Cornielli, Lobke, Jurgen, Bart, Patrick en Monique, zijn alle geplande en ongeplande onderzoeken toch vlot uitgevoerd. De 3D reconstructie van de vetcellen, de in vitro cultuur van de stukjes vetweefsel, de vele liters fixatievloeistof voor alle mogelijke staaltjes, de coupes en kleuringen van alle vetstaaltjes, werden allemaal vlot afgehandeld na een mailtje of een korte bespreking. Bedankt!

Dokter Hostens, Miel, co-promotor, baas (tijdens de GplusE periode), de manier waarop jij met 1000 en 1 dingen tegelijk bezig bent verdient respect. Je motivatie werkt aanstekelijk en de bedrijfsbegeleiding is erop vooruit gegaan sinds je ons toegang hebt gegeven tot de wondere

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wereld van de big data. Als statisticus van de groep kon je me altijd verder helpen met de moeilijkste modellen en de interpretatie hiervan. De reisjes naar de US waren echt geweldig. De bezoeken aan de grote bedrijven in Michigan, Wisconsin, Texas en Kansas hebben mijn enthousiasme voor de melkveesector en de bedrijfsbegeleiding alleen maar aangewakkerd. Het nut van mijn doctoraat ben ik beginnen inzien toen ik begon met het berekenen van rantsoenen. Op het juiste moment wist je me te motiveren om rantsoenen uit te rekenen bij enkele klanten van de bedrijfsbegeleiding en dankzij Bovirant was dit een plezier. Je kwam aandraven met de Hepatic Oxidation Theory van Mike Allen en de zetmeeltheorie van Randy Shaver en sindsdien is de wereld van melkveerantsoenen voor mij opengegaan. De rantsoenmiddagen voor de studenten zijn de ideale momenten om onze kennis over te dragen naar enkele enthousiaste studenten die het nut en belang hiervan inzien en hopelijk kan je deze rantsoenmiddagen nog lang kan voortzetten. Bedankt voor alles en veel succes bij de opvoeding van Leon samen met Kristine!!

Daarnaast wil ik ook de leden van de begeleidings- en examencommissie bedanken. Prof. dr de Kruif, het doet me veel plezier dat u hier vandaag als voorzitter zit. Prof. dr. Geert Janssens, Prof. Dr. Veerle Fievez, Prof. dr. Helga Sauerwein, Dr. Sabine Mann, Prof. Dr. Philippe Bossaert and Prof. Dr. Catherine Delesalle, thank you very much for reading this PhD thesis and your comments and questions during the internal defense. Prof. Delesalle, dankzij uw voorstel om Caro me te laten helpen voor de mRNA expressie studie, is er nog een hoofdstuk bijgekomen in dit doctoraat, super bedankt daarvoor!! Sabine, I was really happy that you accepted the invitation to be part of my exam committee and that you were able to come to my public defense. I need to thank you and Matthias for your hospitality during my stay at Cornell University in the beginning of this year. I really enjoyed our discussions about insulin resistance. Dr. McArt, Jessica, you made it possible for me to visit Cornell University. Thank you for that and hopefully there will be a successful exchange of students between Cornell and Ghent University in the future. I had a wonderful time in Ithaca and met some very nice and famous people in the Dairy Science world. For me it was one of the highlights in my life as a PhD student.

Collega's van het bedrijfsbegeleidingsteam, ik denk dat we de laatste jaren echt een stap vooruit gezet hebben in de bedrijfsbegeleiding. Beoordelen van vruchtbaarheid, uitrekenen van rantsoenen, mee stallen designen, ongelooflijk hoe vlot het allemaal ging. En we konden altijd te raad gaan bij elkaar. Kristof, ik kan me niet meer herinneren wanneer ik je voor het eerst heb gezien, maar je bent één van de beste collega's die ik ooit heb gehad. Toen we begonnen met de bedrijfsbegeleiding op enkele nieuwe bedrijven, nam je rustig de tijd om ons alle mogelijke grafieken uit te leggen. Problemen met de dataverbinding werden vakkundig door jou opgelost (al moest de computer uiteengehaald worden). Ook na de uren hebben we ons kunnen amuseren: de avond in de overpoort dat je je auto helemaal hebt uitgekuist om wat studenten te kunnen vervoeren om naar hun poesjes te gaan kijken, zal me altijd bijblijven. Nu je al een paar maand geen dienst meer doet, zijn de dinsdag- en woensdag-avonden toch niet meer wat ze geweest zijn. Het wordt hoog tijd dat je nog eens komt eten. Veel succes met je nieuwe job, het afwerken van je huis en hopelijk vind je ooit nog ergens wat tijd om je doctoraat af te werken. Mieke, ik heb ervan genoten om op je systeem te werken, soms ging het er wat over of was je wat overgevoelig voor wat ik zei of deed, maar ik had altijd plezier. Bedankt voor de gezamenlijke bedrijfsbegeleidingsbedrijven (we zouden nog eens eentje moeten gaan drinken bij de Robby), voor de namiddagen op de proefhoeve met de studenten en de occasionele glucose tolerantie test. Hopelijk lukt het je om ook iets zinvols te schrijven en kan je je doctoraat binnenkort afwerken. Hannes, succes met je residency en hopelijk lukt het je om enkele leuke artikels te schrijven over de pancreas. Brecht, succes met de opvolging van de bedrijven en als je ooit vragen hebt over rantsoenen, kan je altijd bij mij terecht. Elke, nog veel succes met het schrijven van je artikels en hopelijk vind je wat tijd om verder te gaan met de vet- en leverstaaltjes van mijn koeien, er zit nog een schat aan informatie in verborgen. Sander, ik vind het nog altijd spijtig dat je niet meer bij ons werkt, nu moet ik alleen luisteren naar Liquid Skies van Kay Tracid of kijken naar de videoclip van J'adore hardcore. Geoff, hopelijk verlies je nooit je enthousiasme om ervoor te blijven gaan en komen we elkaar regelmatig tegen op één of ander feestje.

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Collega's van het buitenpraktijkteam, mijn bijdrage was maar van korte duur maar ik heb het ervaren als een uitdaging om elke dienst met een positief saldo te eindigen en aldus het aantal dierlijke klanten van de buitenpraktijk te verhogen ☺. Ik ben blij dat ik in jullie team heb gezeten en met jullie heb samengewerkt: Jef, Marcel, Johan, Bart, Hans, Ameline, Dimitri, Maya, Vanessa, Brecht en Hannes. Jef, bedankt om al de wijsheid die je met ons hebt gedeeld en om al je telefonisch advies als ik van dienst was. Ik stond en sta er nog altijd van te kijken hoe rustig en gestructureerd je te werk gaat. Johan en Bart, bedankt om me een paar keer uit de nood te helpen als ik niet meteen de juiste oplossing vond voor 1 of ander probleem. Bart, ik weet zeker dat de zwaluwen volgend jaar met nog meer gaan zijn als dit jaar en dat Hans als medevoorzitter van het SLF er zal in slagen dat de voortplanting van de zwaluwen nog succesvoller zal zijn als dit jaar. Vanessa, als bureau (3x) – en teamgenoot hebben we elkaars pad verschillende jaren gekruist. Ik ben blij dat ik je nu ook af en toe eens tegenkom op de bedrijven in de bedrijfsbegeleiding die ik van jou heb geërfd. Ik heb heel veel van jou bijgeleerd en vind het knap dat je nu een eigen succesvolle praktijk runt. Maya, mijn eerste week buitenpraktijk als assistent was met jou als student, ik heb me toen enorm goed geamuseerd. Volop keizersnedes (bij oa de 'heks') tot alle blauwe kieltjes op waren en we verplicht terug moesten naar de faculteit. Ik sta ervan versteld hoever je al staat na 1 jaar dienst en ben ontzettend dankbaar dat je me af en toe ook eens uit de nood hebt geholpen, vooral dan tijdens de laatste maanden van mijn doctoraat (het heeft me wat koffiekoeken gekost ☺). Blijf even gemotiveerd verder doen en glimlachen als je de bureau binnenkomt, geniet van de feestjes met de studenten en hopelijk komen we elkaar nog regelmatig tegen! Ria, de moeder van de buitenpraktijk, de ideale schoonmoeder, altijd stond je klaar om ons te helpen, of maakte je tijd om even te luisteren naar onze problemen of je eigen verhaal te vertellen. Voor een klein hongerke konden we altijd een boterhammeke met choco komen halen (die van 't boerinneke vind ik toch de beste). Bedankt voor alles!!

Collega's van het M-team, Anneleen, Ameline, Kristine, Joren, Zyncke, Marina, Lisa, Justine, Sofie, gelukkig zijn jullie er om het progesteron niveau van de vakgroep op peil te houden. Anneleen, bedankt voor alle praatjes in de gang, de keuken, de kliniek, het labo. Toen ik begon met de keizersnedes op de kliniek stond ik ervan te kijken hoe vlotjes jij gedurende de

volledige keizersnede, geen seconde stilte liet vallen. Altijd was er wel wat te zeggen en ik betrap mij erop dat ik na al die jaren keizersnedes juist hetzelfde begin te doen en zeggen als jij toen ☺. Veel succes met je postdoc functie in jullie team en veel plezier nog met Anthony in het nieuwe huisje. Ameline, dank je wel voor alle last-minute dienstwissels en voor de gezellige babbels in het labo, de gang of de keuken. Kristine en Joren, het congres in Kansas City enkele jaren geleden zal me altijd bijblijven als 1 van de beste congressen ooit, zowel op wetenschappelijk als sociaal vlak. Joren, bedankt voor je onvoorwaardelijke hulp bij het uiteensnijden van de koeien. Kristine, hopelijk lukt het jou ook om je doctoraat af te werken op korte termijn, alvast veel succes daarbij en in je nieuwe job.

Collega's van het paardenteam, Jan, Cyriel, Katrien, Kim, Margot, nog altijd ben ik jaloers dat een keizersnede toch een 40 tot 60 minuten langer duurt dan een gewone veulening. Maar desalniettemin heb ik veel respect voor wat jullie elk seizoen weer verzetten van werk. Jan, hopelijk lukt het je ooit om een doctoraat te schrijven. Cyriel, hopelijk vlot het afwerken van de artikels en wordt je niet teveel geremd door de statistische schakel van het proces en kan je je verder specialiseren in de wilde beesten. Katrien, je passie voor het surfen heeft veel gelijkenissen met mijn passie voor het vissen, als het weer eens waaide (vooral dan zuidwest), was je niet te houden omdat je kost wat kost het water op moest. Leuk om te zien dat ik niet de enige was op onze vakgroep die zo geobsedeerd is door een hobby. Margot, ondertussen ex-huisgenootje, het samenleven met ons gedrieën, getweeën, gedrieën en nu terug getweeën, in de bosdreef in Merelbeke, verliep ongelooflijk vlot. Het was een plezier om thuis te komen na enkele keizersnedes en te zien dat het eten klaarstond of vlug nog een pannenkoek met een theetje klaar werd gemaakt om wat op te warmen. Samen vlug iets eten en iets drinken, samen naar een feestje, ik heb ervan genoten. Ik, en Luna ook, gaan je enorm missen, het was een mooie tijd in de bosdreef... Kim, ook wij hebben in de laatste 6 jaar wat dingen meegemaakt, heel wat feestjes (923, TML, Gentse feesten) en samen vanop onze bureau kijken naar de paarden die niet in de van wilden gaan ☺. De drang om de wereld te zien is iets wat we gemeenschappelijk hebben maar ik denk dat jij er de laatste jaren beter in slaagt als ik (daar ga ik dus de komende jaren verandering in brengen).

Sonia, Lynn and Bart, when I needed to talk with somebody outside the team about other things than cows, I came to your desk. Lynn, ook jij hebt meegemaakt wat ik nu bijna 3 jaar geleden heb meegemaakt. Onze gesprekken in die periode hebben me echt geholpen. Veel plezier met het verbouwen van jullie huis en veel succes met het afwerken van je doctoraat!! Sonia, thank you for all the talks, the dinners and the parties. Your Spanish temperament is maybe a little bit confronting for us but for me it was a real pleasure talking with you and I think we, Belgians, should start to act a little bit more Spanish. I wish you good luck in finding a new job on an interesting place on earth. Bart, huisgenootje, ook jij stond altijd klaar om te luisteren naar mijn problemen. Je enthousiasme en volharding tijdens je PhD waren ongelooflijk en je hebt heel mooie resultaten behaald. Bedankt voor de rondrit aan de golf van Mexico en de vele feestjes waar je mee naartoe ging. Nu ik dit doctoraat achter de rug heb, besef ik maar al te goed wat jij hebt doorgemaakt een jaar geleden. Blijf enthousiast doorzetten in je onderzoek en ik ben er zeker van dat je ooit de sleutel tot IVF bij paarden zal vinden.

Onze vakgroep telt teveel mensen om ze allemaal op te noemen, velen zijn al komen te gaan, anderen zijn nieuw: hierbij een poging om wat namen op te sommen (ik ben zeker dat ik er ga vergeten): Catharina, Eline, Valérie, Maaïke (kortom de zwangere/lacterende bureau van de vakgroep), Nerea, Jeroen, Ann, Rubèn, Ruben, Alfonso, Ilse, Bénédicte, Branko, Osvaldo, Pouya, Josine, Kaatje, Maarten, Sarne, Dominique, Davy, Hilde, Eline, Steven, en nog zoveel anderen, bedankt om af en toe dag te zeggen in de gang of voor de meer of minder vlotte conversatie. Catharina, huisbazin, bedankt om de bosdreef aan ons toe te vertrouwen en wat er gebeurd is met die doorgezakte trap zal je vroeg of laat wel eens te horen krijgen (maar niet via mij ☺). Branko, Prof. Atanasov, thanks to you, we had a wonderful time in Macedonia. Osvaldo and Pouya, ladyboy and big boy, as strangers in our team you found each other quite quickly which resulted in a lovely relationship ☺. Pouya, I am still feeling sorry that you made the switch to the horses but hope you have a nice time in the US. Osvaldo, I never thought that we would finalize our PhD at the same moment. I enjoyed seeing you getting nervous when the deadline was approaching. Thank you for the parties and BBQ's during the years that you were here. Hopefully, we can cooperate when we are at the other side of the ocean.

Beste laboranten, Isabel, Petra en Lars, jullie hebben me vaak genoeg zien sukkelen als er een protocol even niet deed wat het moest doen. Ik ben jullie ontzettend dankbaar voor al het werk dat jullie voor mij hebben gedaan!! Isabel en Petra, bedankt voor de insulineoplossingen te maken voor de clamp testen alsook voor het medium voor mijn in vitro proef. Isabel, bedankt om me af en toe ook eens mee te nemen op 1 van je natuuruittappen, het was telkens de ideale moment om mijn hoofd wat leeg te maken en te genieten van de kleine dingen. Je enthousiasme en kennis van alle vogels is oneindig, veel succes nog met de nieuwe job en het kerkuilenproject! Lars, een hoofdstuk in dit boek is te danken aan jou. Bedankt voor de leuke babbel tussen wat analyses door, voor de feestjes, het reisje naar Dubai,...

Daarnaast stond er nog een heel team klaar, zowel in de kliniek als daarbuiten om te helpen waar nodig. Steven, bedankt voor het oplossen van alle computerproblemen. Sandra, bedankt voor alle hulp bij het invullen van de juiste papieren en het regelen van alle ondenkbare zaken. Leila, bedankt voor de hulp langs de financiële kant. Els, bedankt voor alle hulp bij het bestellen van de nodige materialen en producten tijdens mijn proeven. Veronique, bedankt om onderzoek rond af en toe eens uit te kuisen wanneer ik het weer eens vergeten was. Dirk, Willy, Marnik en Wilfried, bedankt om zo goed te zorgen voor mijn proefkoeien destijds. Marnik, bedankt om klaar te staan met hulp waar nodig. Willy, de komende twee jaar zal ik geen schapen komen halen maar daarna ben ik terug vaste klant! Dirk, als je zoon nog eens boilies nodig heeft, laat maar weten.

Heel wat mensen buiten de vakgroep hebben meegeholpen aan het tot stand komen van dit werk. Professor Rehage and Dr. Marian Kusenda from the University of Hannover, thank you very much for sharing and explaining the protocol of the HEC tests. Professor Sauerwein and Christiane Urh, thank you for analyzing the adiponectin samples! Professor Fievez en Erik, bedankt voor de triglyceride bepaling in de vetstaaltjes! Professor Cnop, de wereld van de MINMOD analyse is open gegaan na een bezoekje aan het Erasmus ziekenhuis in Anderlecht. Professor Gerloh, bedankt om me in te leiden in de wereld van de western blots. Lydia, Karel en André van de proefhoeve, jullie hebben me vooral gezien in de eerste helft van mijn doctoraat. Dankzij de PRID proef op jullie koeien heb ik leren tasten en scannen. Op de vakgroep genetica hebben ze me ook vaak zien komen en gaan. Professor Peelman, bedankt om

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me toegang te geven tot jullie labo. Mario, bedankt voor je geduld en je hulp tijdens alle stappen van de genetica studie. Altijd had je wel even tijd om na te gaan wat er goed of fout ging. Ik ben echt blij dat dit deel van het onderzoek ook tot een mooi resultaat heeft geleid. Dit was echter niet mogelijk geweest zonder alle hulp van Caro. Caro, je precisie bij het uitvoeren van de verschillende protocollen, je enthousiasme en nieuwsgierigheid naar de resultaten hebben echt het verschil gemaakt gedurende de laatste analyses voor dit onderzoek. Bedankt voor al je hulp!! Ook de andere mensen op genetica, Ruben, Linda, Dominique, Wenwen, Eline, Sara, bedankt voor jullie hulp of de babbel tijdens het wachten tussendoor.

Bert, zonder jou en je koeien, zou dit doctoraat nooit geweest zijn wat het nu is. Als we proefkoeien nodig hadden om het één of ander op uit te testen, was je altijd bereid om mee te helpen zoeken naar de juiste koeien. Tijdens de bezoeken aan Axelwalle voor stalen te nemen heb ik veel bijgeleerd en je hebt me in het begin vaak genoeg zien klungelen ☺. Mijn rantsoenkennis is ook mede te danken aan jouw kennis en het feit dat er zoveel verschillende voedermiddelen werden gebruikt op Axelwalle. De producties van je koeien zijn hiervan een weerspiegeling. Hopelijk lukt het je om dit ook in de toekomst te bereiken en te overtreffen.

Beste klanten van de buitenpraktijk en bedrijfsbegeleiding, bedankt om zo geduldig te zijn en om de studenten de kans te geven dit bijzondere beroep te leren. Zonder jullie zou er geen buitenpraktijk en bedrijfsbegeleiding zijn en dat zou een zeer groot verlies zijn voor de diergeneeskundige wereld. De samenwerking met enkele klanten van de bedrijfsbegeleiding was wat intensiever dan met andere klanten. Erwin en Wivine, het was altijd enorm gezellig om bij jullie op bedrijfsbezoek te komen. Samen met Kristof hebben we hier vruchtbaarheidsresultaten behaald waarvan ik dacht dat deze de dag van vandaag onmogelijk waren. Pieter en Annick, de nieuwe stal stond er sneller dan ik had verwacht en ik weet zeker dat indien jullie blijven volhouden, de resultaten er ook zullen komen. Wim en Leen, ook bij jullie heeft de nieuwe stal grote veranderingen teweeg gebracht. Alhoewel mijn eerste bedrijfsbezoeken bij jullie niet positief waren geëindigd, ben ik enorm blij dat jullie me toch nog een kans hebben gegeven en ben benieuwd naar de resultaten eens jullie gaan starten met melken op de nieuwe maiskuil (met goede haksellengte ☺). Kris en Erika, het uitzicht tijdens het tasten op jullie bedrijf is super! Tijdens de jaren dat ik bij jullie langskwam, is ook hier het

één en ander verandert en ik ben er zeker van dat als jullie zo blijven voortdoen, de resultaten ook stilaan gaan verbeteren. Jullie allemaal wil ik bedanken voor de vele vragen, het was telkens een uitdaging om het juiste antwoord te formuleren of te gaan opzoeken en te focussen op de belangrijke dingen en jullie te overtuigen van bepaalde zaken. Het optimaliseren en uitrekenen van de rantsoenen, de toename van de melkproducties en de verbetering van de vruchtbaarheidsresultaten waren voor mij de drijfveer om er volop te blijven voor gaan!! Veel succes in de toekomst !!

Beste studenten, ik heb ervan genoten om jullie uitleg te geven over alles wat met koeien te maken heeft. Zonder jullie waren de keizersneden maar half zo leuk. Jullie enthousiasme om eindelijk iets praktisch bij te leren was enorm motiverend. Hopelijk blijven de rantsoenmiddagen doorgaan en blijven jullie even enthousiast komen. Jullie feestjes waren voor mij de ideale momenten om me even volledig te laten gaan. Het laatste jaar op de faculteit is het beste jaar van de studies (zeker voor de optie herkauwers), ik ben blij dat ik elk jaar toch weer heb kunnen meegenieten van jullie laatste jaar gevoel. Janine, jij bent 1 van de studenten waar ik enorm veel plezier mee heb gehad: de feestjes, de voetbalmatchen tijdens het WK 2014 (dat Duitsland dan ook won ☺), rantsoenen uitrekenen op een blad (gelukkig was je snel overtuigd van het nut van Excel) of gewoon wat bijpraten. Ik hoop dat het je lukt om je droom te verwezenlijken en dan kom ik zeker eens op bezoek!! Beste studenten van de Amerika reis van 2015, Evi, Lieze, Nicky, Jana, Jordy, Jacqueline, Jonas, Tuur, Ralph, Raf, Alexander, Michiel en Matthias, ik had nooit gedacht dat dit mogelijk zou zijn, maar jullie hebben het toch maar voor elkaar gekregen. Op het moment dat ik eigenlijk volop moest schrijven aan mijn artikels, planden jullie een rondreis door Michigan en Wisconsin. Ik heb genoten van jullie enthousiasme bij het zien van de grote bedrijven en al de koeien die ginder rondlopen!

Fille, Martin en Hans, kameraden, als ik weer eens enkele weken teveel gefocust was op het werk, kan een weekje vissen of een avondje weg met jullie me weer in balans brengen. Om de 1 of andere reden beleven we altijd unieke momenten als we samen op pad gaan: de kerstmarkt in Brussel, het nachtleven van Berlijn, TML, een avondje Utrecht, de bergen van Lapland, onze visserij op het machtige Lac du Der, Cassien of gewoon samen iets eten. Stuk voor stuk momenten uit mijn leven die me voor altijd gaan bijblijven en die ik absoluut niet had

willen missen. Ik ben er zeker van dat er nog veel van dergelijke momenten gaan volgen. Stephanie, merci om er voor ons allemaal te zijn. Als enige vrouw in het gezelschap, is het niet gemakkelijk om ons tegen te houden als we weer even het zot in de kop hebben. Maar we geraken altijd wel heelhuids terug thuis op de één of andere manier ☺. Het is weer al even geleden dat we samen nog eens een stapje in de wereld hebben gezet, maar de komende weken zijn er kansen genoeg om hier verandering in te brengen...

Beste familieleden, jullie zijn te talrijk om allemaal op te noemen en te bedanken. De laatste jaren hebben jullie me niet vaak gezien op familiefeestjes omwille van het feit dat ik of van dienst was of bezig was met proeven. Toch een woordje van dank aan diegene die me echt hebben geholpen de laatste jaren. Meter, altijd staat je deur open om even binnen te springen en ben je toch benieuwd naar wat we allemaal aan het uitsteken zijn. Marcel en Peter, duizend maal dank om op Luna te passen als ik voor een paar dagen of weken weg moest en voor de theetjes met bijbehorende babbel. Marcel, bedankt voor de tips bij het opvoeden van Luna, ze is echt een geweldig hondje en ik ben zeker dat dit met Pipa in de toekomst ook zal lukken!!

Lobke, Rien en Jaan, binnenkort zijn we allemaal het huis uit, onze eigen weg op. De één gaat al wat verder dan de ander, maar we blijven 1 familie en we kunnen altijd bij elkaar terecht voor eender wat. Lobke en Jeroen, de deur van jullie huis staat altijd open zoals dat ooit ook bij ons thuis het geval was. Even binnenspringen om wat te spelen met de jongens was altijd ideaal om alles terug op een rijtje te zetten en de dingen des levens wat te relativeren. Dante, Nand en Lasse, jullie zijn drie toffe kerels!! Rien, bedankt voor de lay out van dit doctoraat. Begin september had ik hiervoor geen geduld en geen tijd meer om dat nog op een deftige manier te doen. Succes met het opwekken van de groene energie in de bio-ethanol fabriek die je aan het bouwen bent, hopelijk zonder al teveel problemen. Jaan, sinds een paar weken ben je ook begonnen aan je eerste job in je carrière als pol en soccer, en heb je ondertussen ook al menig kilometer file achter de rug. Binnenkort ga je ook het huis uit, veel succes met je nieuwe appartement!!

Mams, geen groter verlies dan jou te moeten missen op deze dag en op alle andere dagen die al gepasseerd zijn of nog gaan komen. Ongelooflijk hoe snel en oneerlijk het is gegaan, nu bijna drie jaar geleden op kerstavond begon het ondenkbare ... Niemand verdiende dit, en zeker jij

niet, maar toch is het gebeurd. Moest je er nog zijn, dan zou je zien hoe mooi de krulletjes van Lasse zijn, hoe goed de rapporten van Dante zijn en hoe Nand overal op en over klautert om kapoenenstreken uit te halen. Dan zou je ze gaan ophalen van school en konden we allemaal samen nog eens eten, Lobke en Rien die thuiskomen van het werk en Jaan die terugkomt van de les (en ondertussen ook al van het werk), allemaal rond 6 uur omdat we weten dat je toch altijd genoeg lekker eten klaarmaakt... Het leven gaat voort, de tijd staat niet stil,... Bedankt voor alles wat je voor mij gedaan hebt.

Beste allen, bedankt!!

Jenne

~ In the end, we will only regret the things we didn't do ~



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